AFRL-HE-WP-TR-2003-0077

UNITED STATES AIR FORCE RESEARCH LABORATORY

IN VITRO RAT HEPATOCYTE TOXICITY AND BACTERIA GENOTOXICITY EVALUATION OF HIGH ENERGY CHEMICALS FOR REPLACEMENT OF HYDRAZINE

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December 2002
Final Report - December 1999 - December 2001

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AFRL-HE-WP-TR-2003-0077

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

Mark M. Hoffman

MARK M. HOFFMAN
Deputy Chief, Deployment and Sustainment Division
Air Force Research Laboratory

REPORT DOCUMENTATION PAGE	Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2002	Final Report - December 1999 - December 2001

4. TITLE AND SUBTITLE
In Vitro Rat Hepatocyte Toxicity and Bacteria Genotoxicity Evaluation of High Energy Chemicals for Replacement of Hydrazine

5. FUNDING NUMBERS
Contract F33615-00-C-6060
PE 61102F
PR 2312
TA 2312A2
WU 2312A205

6. AUTHOR(S)
Hussain, S., Sharma, S., Gao, P., Mattie, D., Frazier, J.
* **

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Human Effectiveness Directorate **
Air Force Research Laboratory
Wright-Patterson AFB, OH 45433-7400

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
ManTech Environmental Technology, Inc.*
PO Box 31009
Dayton, OH 45437-0009

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
AFRL-HE-WP-TR-2003-0077

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

In an effort to develop methods to predict the toxicological response of newly synthesized chemicals that are of interest to the US Air Force, in vitro rat hepatocyte toxicity and bacteria (Salmonella) genotoxicity assays were performed on thirteen high-energy chemicals (HEC) that were selected as potential replacements of hydrazine. The HECs were primarily hydrazine derivatives (hydrazinium nitrate, HZN; 2-hydroxyethylhydrazine nitrate, HEHN; diethyl hydrazine nitrate, DEHN; 1,4-dihydrazine nitrate, DHTN; methylhydrazine nitrate, MHN; diaminoguanidine nitrate, DAGN; 2,2-dimethyltriazanium nitrate, DMTN; nitroaminoguanidine nitrate, NAGN), amino containing compounds (ethanolamine mitrate, EAN; histamine dinitrate, HDN; methoxylamine nitrate, MAN), and triazole containing compounds (1,2,4-triazole nitrate, TN; 4-amino-1,2,4-triazole nitrate, ATN). The results in hepatocytes showed a dose dependent decrease in mitochondrial activity (MTT), increase in lactate dehydrogenase (LDH) leakage, and depletion of GSH levels. Responses to hydrazine were used as reference values for ranking the other HECs. According to the MTT assay, the hydrazine-containing compounds are the most toxic (HZN > DEHN > DHTN > MHN > HEHN > DAGN > NAGN), amino-containing compounds displayed medium toxicity (HDN > EAN > MAN) and triazole-containing compounds exhibited low toxicity (DMTN > ATN > TN). Results of genotoxicity (Ames assay) on 10 of the 13 HECs indicated that HZN, DHTN and MAN were mutagenic. DHTN was a direct mutagen causing AT and GC base-pair substitutions and frameshift mutations.

14. SUBJECT TERMS	15. NUMBER OF PAGES
hydrazinium nitrate, HZN 2-hydroxyethylhydrazine nitrate, HEHN diethyl hydrazine nitrate, DEHN	83 16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Standard Form 298 (Rev. 2-89) (EG)
Prescribed by ANSI Std. 239.18
Designed using Perform Pro, WHS/DIOR, Oct 94

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PREFACE

This document is part of the final reporting process for the Air Force Research Laboratory/Operational Toxicology Branch (AFRL/HEST) project "Toxicity of High Energy Chemicals" (JON# 2312A205). This preliminary toxicology report is divided into two sections. Section I provides information on *in vitro* hepatocyte toxicity evaluations. These investigations were performed at AFRL/HEST, Wright Patterson Air Force Base, Ohio. Section II provides information on genotoxicity evaluations. These studies were performed by the Cellular and Molecular Toxicology Program, ManTech Environmental Technology, Inc., Research Triangle Park, North Carolina. The Toxicity of High Energy Chemicals research project was funded by the Air Force Office of Scientific Research (AFOSR) and was initiated in April 1999, under Department of the Air Force Contract No. F41624-96-C-9010 and completed under Contract No. F33615-00-C-6060. Dr. Richard R. Stotts served as the Contracting Officer's Representative for the U.S. Air Force. Darol E. Dodd, Ph.D., served as Program Manager for ManTech Geo-Centers Joint Venture.. Authors would like to acknowledge TSgt Gerri Miller, TSgt Michelle Curran and Darin Minnick for their excellent technical support.

IN VITRO RAT HEPATOCYTE TOXICITY AND BACTERIA GENOTOXICITY EVALUATION OF HIGH ENERGY CHEMICALS FOR REPLACEMENT OF HYDRAZINE

SECTION I. TOXICOLOGICAL ASSESSMENT OF HIGH ENERGY COMPOUNDS: *IN VITRO* HEPATOCYTE TOXICITY

Prepared by Saber Hussain, PhD (Operational Toxicology Research, ManTech Environmental Technology, Inc., Dayton, OH), David R. Mattie, PhD, and John M. Frazier, PhD (AFRL/HEST, Wright-Patterson AFB, OH).

SUMMARY

In an effort to develop methods to predict the toxicological response of newly synthesized chemicals that are of interest to the U.S. Air Force, we have evaluated the *in vitro* toxicity for thirteen high-energy chemicals (HEC) in rat hepatocytes. Hydrazine is an aircraft fuel and propellant used by the US Air Force. Due to its toxicity the Propulsion Directorate of the Air Force Research Laboratory (AFRL/PR) has synthesized a series of high-energy chemicals (HECs) as potential hydrazine replacements. The HECs are comprised primarily of hydrazine derivatives (hydrazinium nitrate, HZN; 2-hydroxyethylhydrazine nitrate, HEHN; diethyl hydrazine nitrate, DEHN; 1,4-dihydrazine nitrate, DHTN; methylhydrazine nitrate, MHN; diaminoguanidine nitrate, DAGN; 2, 2-dimethyltriazanium nitrate, DMTN; nitroaminoguanidine nitrate, NAGN), amino containing compounds (ethanolamine nitrate, EAN; histamine dinitrate, HDN; methoxylamine nitrate, MAN), and triazole containing compounds (1,2,4-triazole nitrate, TN; 4-amino-1,2,4-triazole nitrate, ATN). The current study was undertaken to examine the toxicity of HECs in rat (male Fischer 344) primary hepatocytes *in vitro*. The effects of short-term exposure (4 hours) of hepatocytes to HECs were investigated with reference to viability, mitochondrial function, reactive oxygen species generation, reduced and oxidized glutathione (GSH and GSSG). The results showed a dose dependent decrease in mitochondrial activity (MTT), increase in lactate dehydrogenase (LDH) leakage, and depletion of GSH levels. Responses to hydrazine were used as reference values for ranking the other HECs. According to the MTT assay, the hydrazine-containing compounds are the most toxic (HZN > DEHN > DHTN > MHN > HEHN > DAGN > NAGN), amino-containing compounds displayed medium toxicity (HDN > EAN> MAN) and triazole-containing compounds exhibited low toxicity (DMTN>ATN>TN). In conclusion, based on these biochemical data, the chemicals were classified into three categories: higher toxicity (hydrazine containing compounds), medium toxicity (amino containing compounds), and lower toxicity (triazole containing compounds).

INTRODUCTION

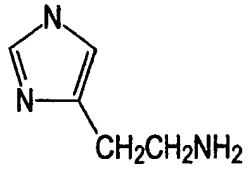
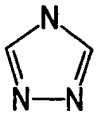
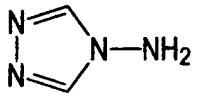
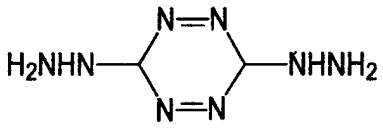
Hydrazine is a highly reactive chemical that has been used as a propellant by the US Air Force. Besides it's application as a propellant and fuel in aircraft, hydrazine has a wide range of uses, including corrosion inhibitors, photographic materials and drugs. Several hydrazine derivatives occur naturally in tobacco and mushrooms, some are herbicides, and others have been shown to be pharmacologically active. Hydrazine and its derivatives enter the environment primarily from aerospace emissions and from manufacturing facilities although exposure also occurs as a metabolite of the drugs isoniazid (an antitubercular agent) and hydralazine (an antihypertensive agent) (Delaney and Timbrell, 1995). Toxic effects due to exposure to hydrazines include liver damage (Kleineke *et al.*, 1979), hypoglycemia, disorders of the central nervous system (Lightcap *et al.*, 1995), interference with intermediary metabolism (Moloney and Prough, 1983) and carcinogenicity (Bosan *et al.*, 1987; Wald *et al.*, 1984).

Several investigators have reported on the toxicity of hydrazine *in vivo* and *in vitro*. Hydrazine exposure leads to ATP depletion and megamitochondria formation *in vivo* (Kerai and Timbrell 1997; Preece *et al.*, 1990; Wakabayashi *et al.*, 2000). Hydrazine inhibits the mitochondrial enzyme succinate dehydrogenase (Ghatineh *et al.*, 1992), which subsequently reduces mitochondrial function. Hydrazine also produces toxicity by interfering with a number of metabolic processes such as gluconeogenesis (Kleineke *et al.*, 1979) and glutamine synthetase (Willis 1966; Sendo *et al.*, 1984; Kaneo *et al.*, 1984).

The disappearance of hydrazine from hepatic microsomes suggested that hydrazine was oxidized by the cytochromes P450, although the product was not identified (Jenner and Timbrell, 1994). A study aimed to ascertain the role of P450 isozymes in the toxicity of hydrazine using rat hepatocytes *in vitro* suggested that metabolism by all three P450 isozymes leads to detoxification and that the cytotoxicity of hydrazine could be due to the parent compound (Delaney and Trimbel, 1995).

The US Air Force continues to evaluate alternative aerospace propellants. In view of hydrazine's toxicity, a series of thirteen high-energy chemicals (HEC) was synthesized by the Propulsion Directorate of the Air Force Research Laboratory, CA (Table I-1). In order to maintain a safe working environment, it is necessary to develop reliable, rapid and inexpensive methods for predicting health risks of newly developed chemicals. The aim of this study was to examine the toxicity of these HEC in primary hepatocytes *in vitro* with reference to viability, mitochondrial function, and redox status of the cells. The toxicological profiles of these chemicals will assist in the design and optimization of chemicals for new propellants.

TABLE I-1: PROPOSED HIGH-ENERGY CHEMICALS (HEC)

Chemical Name	Abbreviation	Neutral Species	Category
Hydrazinium nitrate	HZN	NH_2NH_2	hydrazine
2-Hydroxyethylhydrazinium nitrate	HEHN	$NH_2NHCH_2CH_2OH$	hydrazine
1,2-Diethylhydrazinium nitrate	DEHN	$CH_3CH_2NHNHCH_2CH_3$	hydrazine
Methylhydrazinium nitrate	MHN	CH_3NHNH_2	hydrazine
Diaminoguanidine nitrate	DAGN	$NHC(NHNH_2)_2$	hydrazine
Ethanolamine nitrate	EAN	$NH_2CH_2CH_2OH$	amine
Histamine dinitrate	HDN		amine
Methoxylamine nitrate	MAN	NH_2OCH_3	amine
1,2,4-Triazole nitrate	TN		triazole
4-Amino-1,2,4-Triazole nitrate	ATN		triazole
2,2-Dimethyltriazanium nitrate	DMTN	$[(NH_2)_2N(CH_3)_2]^+$	quaternary ammonium salt
1,4-Dihydrazinotetrazine nitrate	DHTN		hydrazine
Nitroaminoguanidine nitrate	NAGN	$NH_2NHC(NH)NHNO_2$	hydrazine

METHODS

Chemicals

Collagenase was obtained from Boehringer-Mannheim Biochemicals (Indianpolis, IN). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-nicotinamide-adenine dinucleotide-reduced (NADH), 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA), reduced glutathione (GSH), insulin/transferrin/sodium selenite (ITS) additive, gentamicin, and dexamethasone were purchased from Sigma Chemical Company (St. Louis, MO). Chee media was obtained from Gibco (Grand Island, NY). All HEC were supplied from the Propulsion Directorate of the Air Force Research Laboratory, Edwards Air Force Base, CA. These compounds may be categorized as hydrazine-based, amine-based, triazole-based, and a quaternary ammonium salt as shown in Table I-1.

Animals

Male Fischer 344 rats (225-300 g) were obtained from Charles River Laboratories (Wilmington, MA). Rats were anesthetized with 1 mL/kg of a mixture of ketamine (70 mg/L; Parke-Davis, Moris Plains, NJ) and xylazine (6 mg/L; Mobay Corp., Shawnee, KS) prior to undergoing liver perfusion. All animals used in this study were handled in accordance with the principles stated in *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

Liver Perfusion, Hepatocytes Enrichment and Culture

Fischer 344 rat livers were perfused, and hepatocytes were isolated and enriched by the two-step Seglen procedure (Seglen, 1976) with minor modifications as previously described (DelRaso and Frazier 1999). Chee media for perfusion (pH 7.2) were supplemented with 15 mM HEPES, washout medium was supplemented with heparin (2.0 U/mL) and EGTA (0.5 mM), and digestion medium was supplemented with 500 mg/L collagenase. Viable primary rat hepatocytes were enriched by low speed centrifugation (500 x g) for 3 min. Typically the yield of isolated hepatocytes was from 300 to 400 million cells with viability ranging from 85 to 95% as determined by trypan blue dye exclusion. For cell culture studies, suspensions of primary hepatocytes were adjusted to a cell density of 1.0×10^6 cell/mL in Chee culture medium (pH 7.2) supplemented with HEPES (10 mM), insulin/transferrin/sodium selenite solution (5 mg/L, 5 mg/L, 5 μg/L), gentamicin (50 mg/L), and dexamethasone (0.4 mg/mL). Cells were seeded in either 96-well (4×10^4 cells/well) or 6-well (1.0×10^6 cells/well) culture plates previously coated with rat tail collagen, 1.0 μg/well or 25 μg/well, respectively. After 4 h of incubation in a 5% CO_2 incubator at 37°C to allow for attachment, hepatocytes were re-fed with Chee culture medium lacking dexamethasone. Hepatocytes were cultured for an additional 21 h before treatment as indicated in the experimental schedule (see Figure I-1).

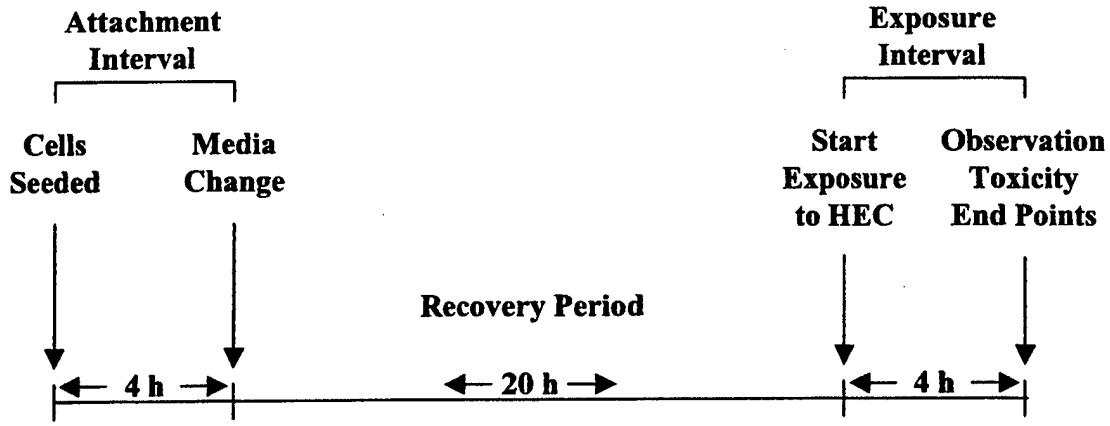


Figure I-1: Schedule for Culturing and Dosing of Primary Hepatocytes. Fischer 344 rat livers were perfused, and hepatocytes were isolated and enriched as described in Material and Methods. After 4 h of incubation to allow for attachment, hepatocytes were re-fed with Chee culture medium and incubated for a further 20 h prior to HEC exposure. Hepatocytes were exposed to hydrazine for 4 h and biochemical evaluations were conducted immediately at the end of the exposure.

Treatment

Primary rat hepatocytes were treated with various concentrations of HEC dissolved in Chee culture media. The cells were exposed to HEC for 4 h (Figure I-1). A number of toxicity end points were evaluated at the end of the 4 h incubation period.

Mitochondrial Function

Mitochondrial function was determined spectrophotometrically by measuring the degree of mitochondrial reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by succinic dehydrogenase (Carmichael *et al.*, 1987). Following treatment, cells were washed and incubated at 37°C in Chee media containing 0.05% MTT for 30 min. At this time the media containing MTT was removed and the colored product (formazan) extracted from the cells in acidified isopropanol and assayed with a spectraMAX plus microplate reader (Molecular Devices, Sunnyvale, CA.).

LDH Leakage

LDH leakage indicates loss of cellular viability because membrane damage that results in LDH leakage is generally considered irreversible. LDH leakage was assessed by measuring the activity of LDH in the cells and released into the media (Moldeus *et al.*, 1978). After treatment, the media was removed from the culture plate and placed on ice. The plates were washed with

cold PBS followed by addition of 1 ml of a 0.5% solution of Triton X-100. The cells were placed on ice for 30 min at which time the solution and cellular debris were carefully removed and vortexed in 2 ml sample vials. Aliquots (10 µl) of the media or detergent solution were then assayed in phosphate buffer containing 0.2 mM NADH and 1.36 mM pyruvate by monitoring the loss rate of NADH absorption at 340 nm with a spectraMAX Plus microplate reader (Molecular Devices, Sunnyvale, CA). The percent of activity in the media was then calculated by dividing the amount of activity in the media by the total activity.

Reduced Glutathione

Glutathione (GSH) is a ubiquitous sulfhydryl-containing molecule in cells that is responsible for maintaining cellular oxidation-reduction homeostasis. Monitored changes in GSH homeostasis are an indication of cell damage. Reduced glutathione (GSH) were measured according to the Glutathione Assay Kit from Cayman Chemical Company, Ann Arbor, MI. The assay is based on enzymatic recycling method, using glutathione reductase and DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) as described by Tietze (1969).

ROS Generation

ROS generation was determined by the method described by Wang and Joseph (1999). Cells were incubated with 100 µM of 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA), in Chee media under standard culture conditions for 30 min prior to chemical treatment. The DCFH-DA is cell permeable and can be oxidized into fluorescent product 2,7-dichlorofluorecein. After the media containing DCFH-DA was removed, the cells were washed and treated with test chemicals in Chee media for 4 hours. At the end of the exposure, the cells were washed with PBS and fluorescence of the cells from each well was measured in a spectraMAX (Molecular Devices, Sunnyvale, CA.) multi-well fluorescence plate reader at excitation 485 nm and emission at 530 nm.

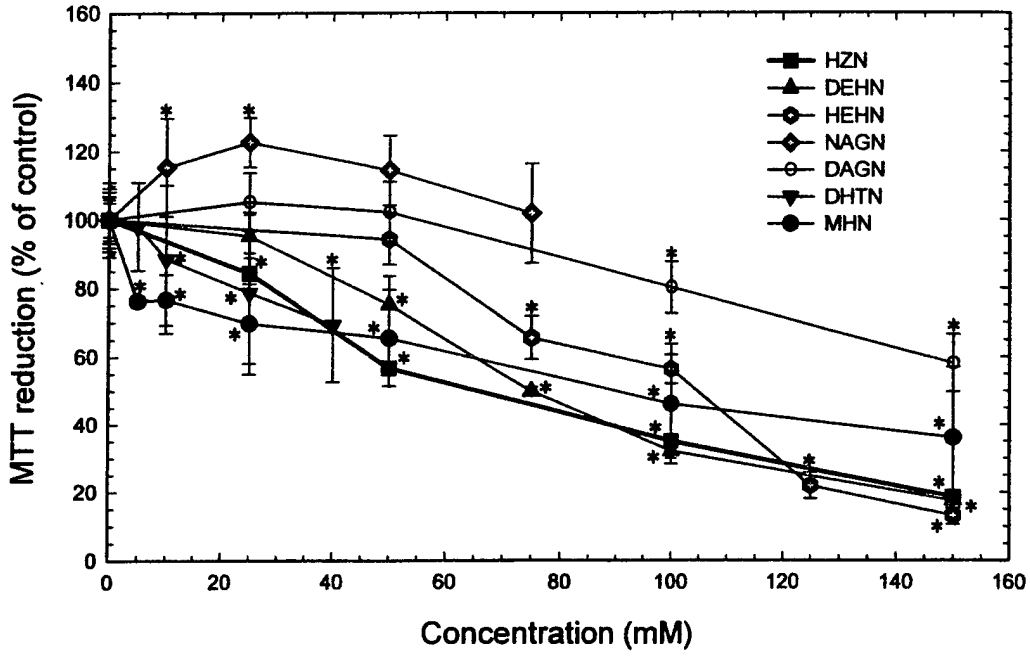
RESULTS

In vitro toxicity of HEC

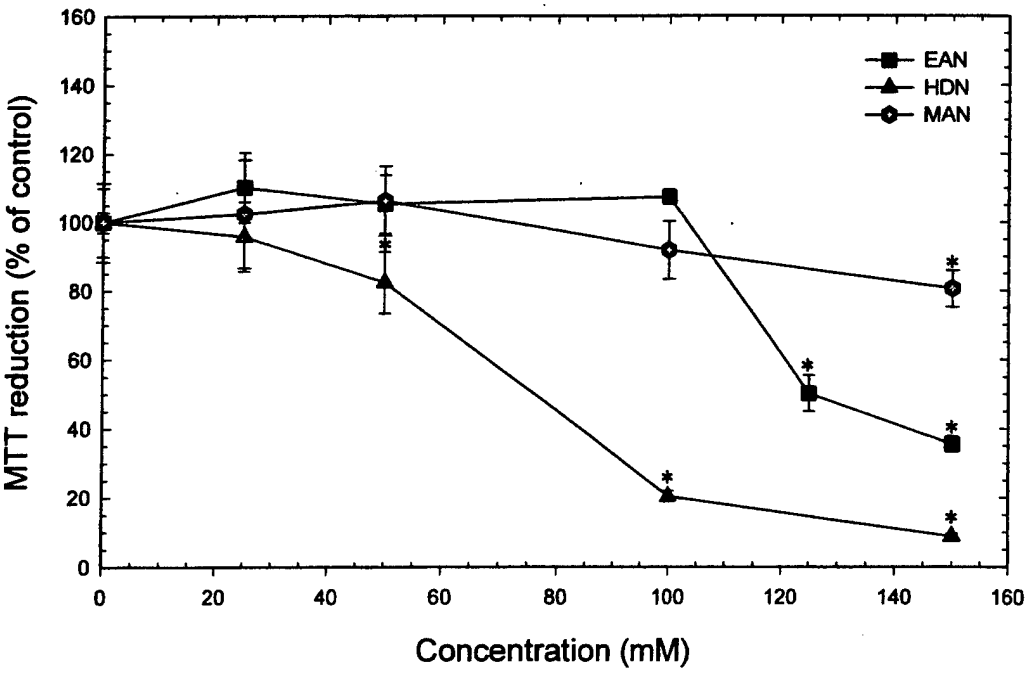
The MTT assay was used to assess the effects of HEC on mitochondrial function of rat hepatocytes. Figure I-2 shows that mitochondrial function of hepatocytes decreases in a dose-dependent manner with increasing HEC concentration. Hydrazine-containing compounds (HZN, HEHN, DEHN, MHN, DHTN, DAGN, and NAGN) reduced mitochondrial function in a concentration-dependent manner. Amino-containing compounds (EAN, HDN, and MAN) displayed toxicity at higher doses except for HDN, which showed toxicity at 50 mM. Triazole containing compounds (TN, ATN, and DMTN) did not exhibit significant toxicity even at the highest dose (150 mM). According to the MTT assay, the hydrazine-containing compounds are

the most toxic, the amino-containing compounds displayed medium toxicity, and the triazole-containing compounds exhibited low toxicity.

I-2A



I-2B



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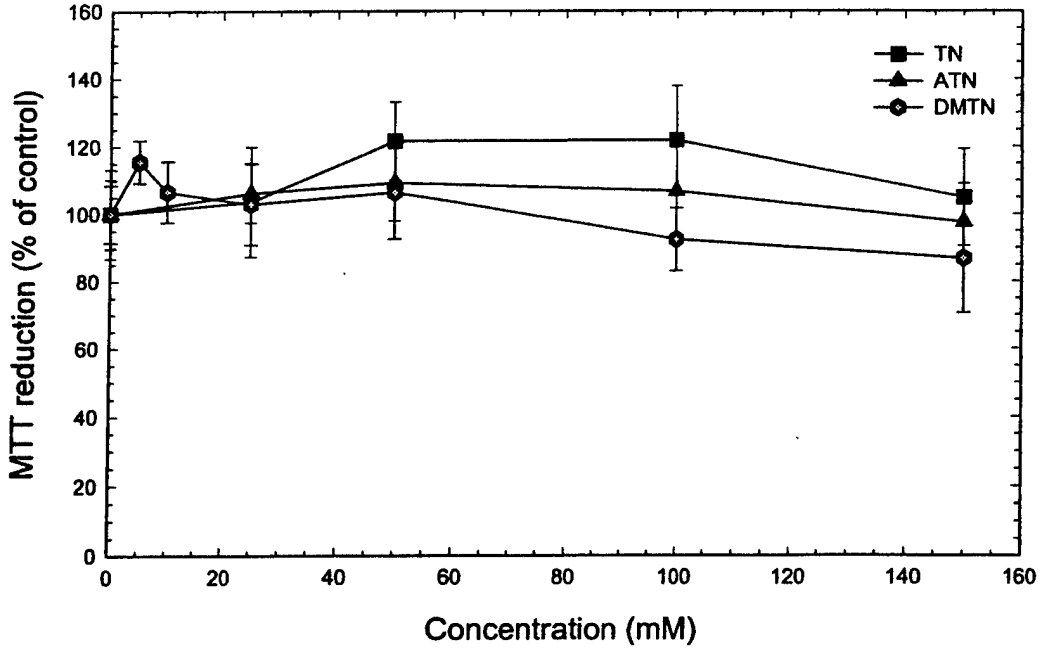
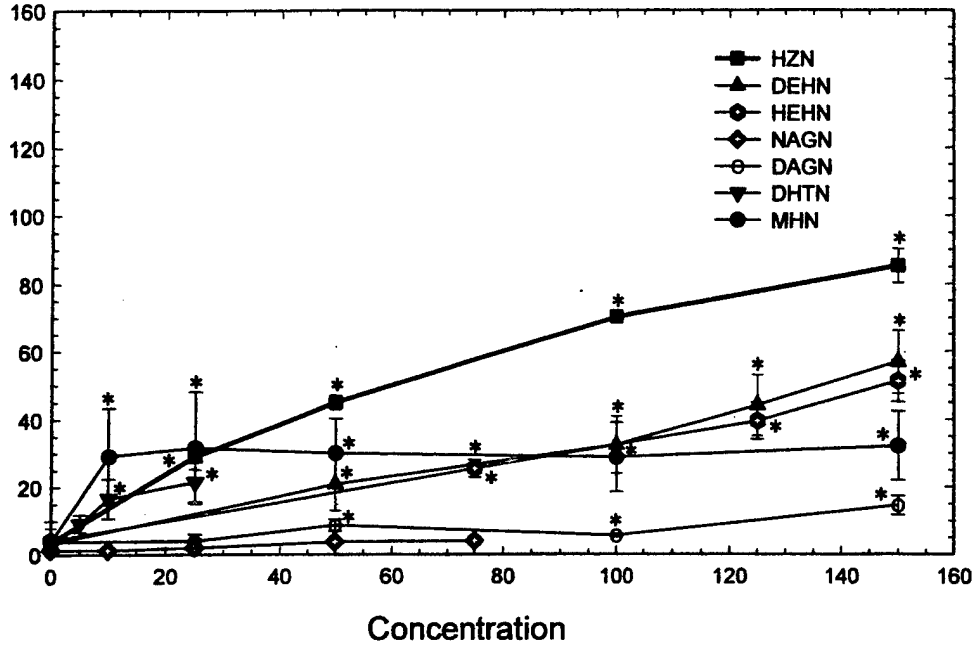


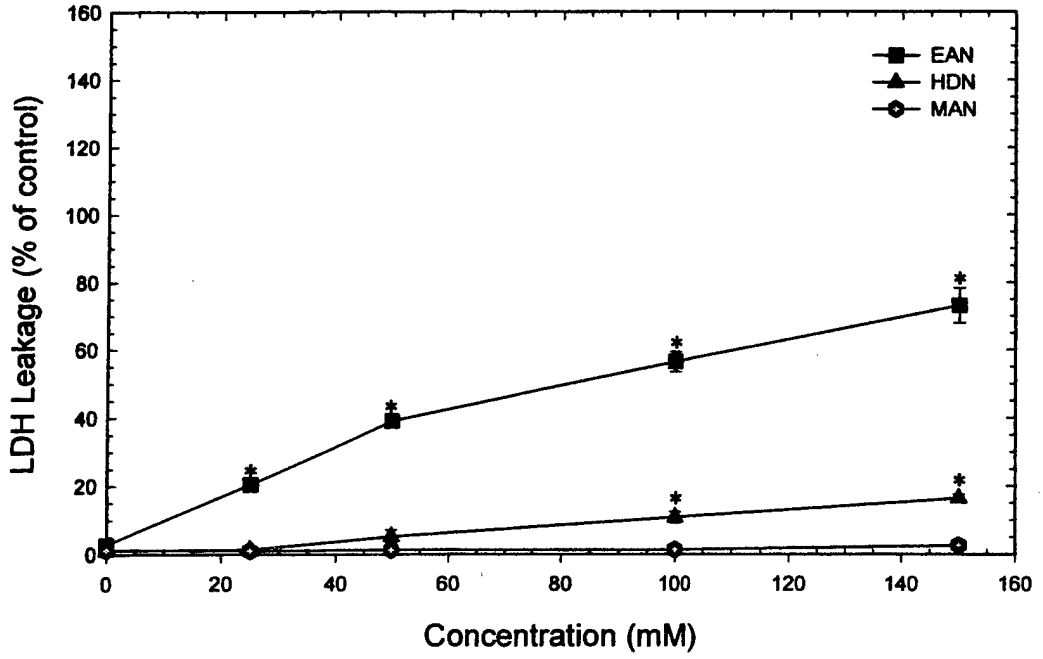
Figure I-2. Effect of HEC on Mitochondrial Function of Hepatocytes.
MTT reduction is plotted as a function of HEC dose for hydrazine-based HEC in (A), for amine-based HEC in (B) and for triazole-based HEC (C). Primary hepatocytes were treated with different concentrations of HEC for 4 h. At the end of the incubation period, mitochondrial function was determined by the MTT assay as described in the Materials and Methods section. The data are expressed as means ± SD of three independent experiments from three different rats. (*) indicates a statistically significant difference compared to controls ($p < 0.05$).

The viability of rat hepatocytes was evaluated by measuring the leakage of LDH into the media. Figure I-3 indicates that 4-h exposure to HEC produced a dose-dependent increase in LDH leakage into the media. HZN appears to be more toxic as it induced 80-90% LDH leakage at 150 mM HZN. The EC_{50} value for HZN is 62 mM. The trend of toxicity as assessed by LDH leakage follows the same trend seen in the MTT assay except that EAN produced higher toxicity compared to HDN and MAN. LDH leakage showed that the order of toxicity as follows: hydrazine-containing compounds > amino-containing compounds > triazole-containing compounds.

I-3A


HZN
DEHN
HEHN
NAGN
DAGN
DHTN
MHN
Concentration

I-3B


EAN
HDN
MAN
LDH Leakage (% of control)
Concentration (mM)

I-3C

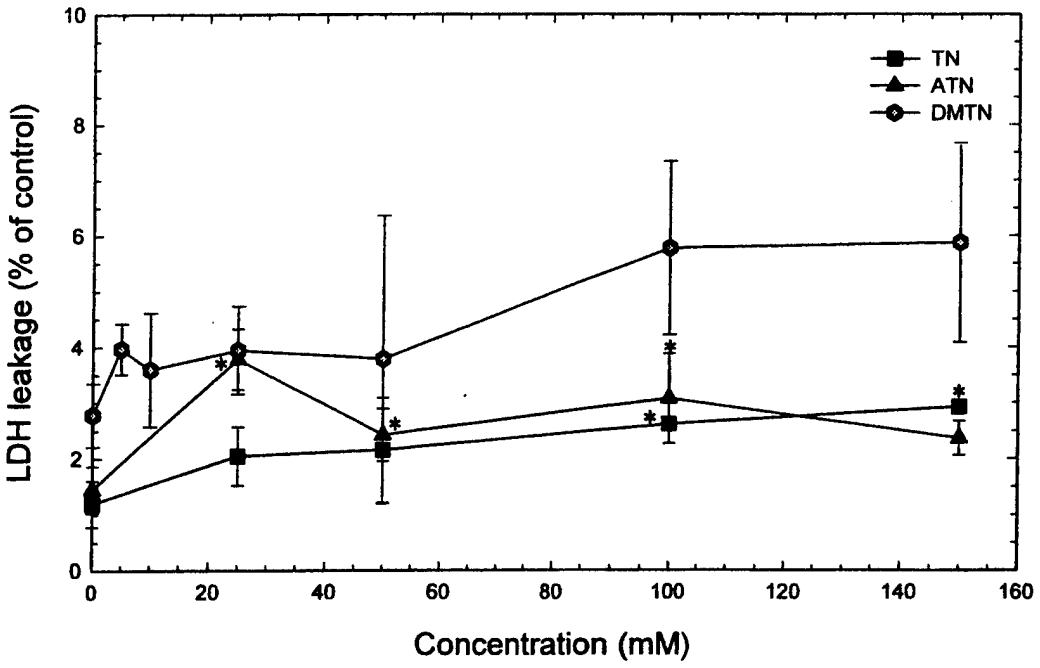
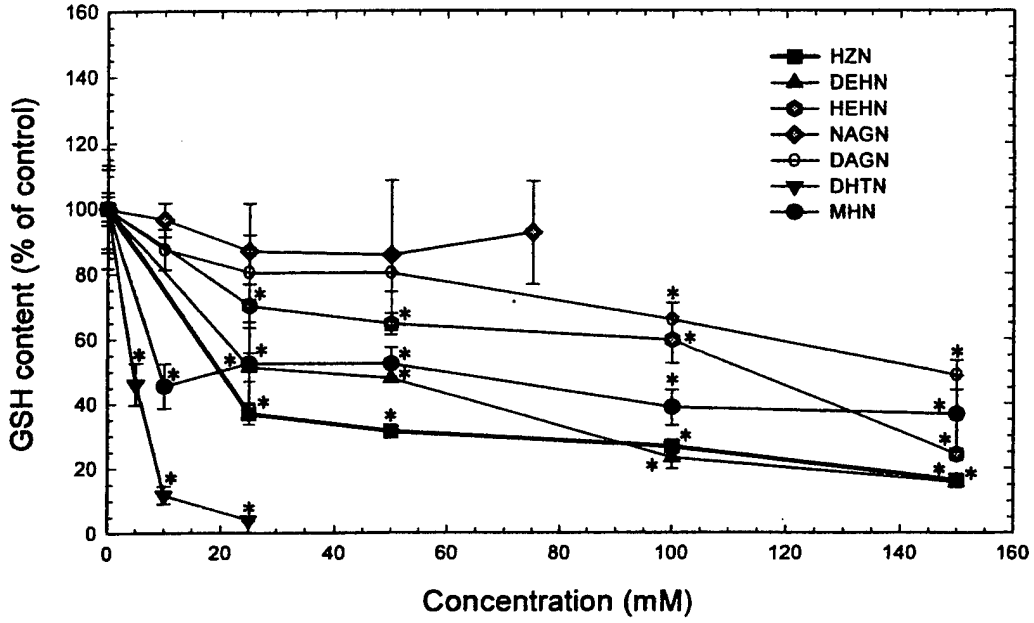


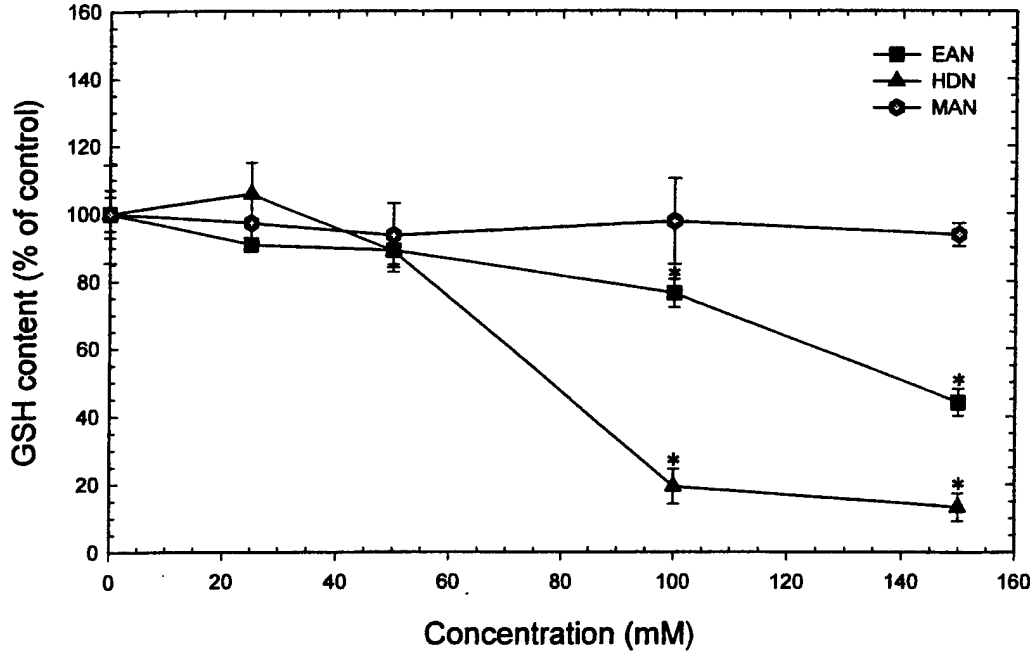
Figure I-3. Effect of HEC on LDH Leakage of Rat Hepatocytes.
LDH leakage into media is plotted as a function of HEC dose for hydrazine-based HEC in (A), for amine-based HEC in (B) and for triazole-based HEC (C). Primary hepatocytes were treated with different concentrations of HEC for 4 h. At the end of the incubation period, LDH leakage was determined by the LDH assay as described in the Materials and Methods section. The data are expressed as means ± SD of three independent experiments from three different rats. (*) indicates a statistically significant difference compared to controls ($p < 0.05$).

In cells, glutathione (GSH) is a ubiquitous sulfhydryl-containing molecule that is responsible for maintaining cellular oxidation-reduction homeostasis. GSH protects cells against damage by scavenging highly reactive free radicals that can interact with critical cellular components. Monitored changes in GSH homeostasis are therefore an indication of cell damage. A dose-dependent decrease in GSH was found in HEC-treated hepatocytes as seen in Figure I-4. GSH levels were measured in control and HEC-exposed cells after 4 hours of exposure. DHTN was found to reduce considerably GSH levels to 4% at 25 mM. A large depletion (70%) of GSH resulted from low dose (25 mM) of HZN followed by subsequent decreases at 50, 100 and 150 mM. The order of toxicity of hydrazine-containing compounds in reducing GSH levels are DHTN> HZN> DEHN> MHN > HEHN > DAGN > NAGN. Based on GSH assay, the most toxic chemical is DHTN.

I-4A


GSH content (% of control)
Concentration (mM)
HZN
DEHN
HEHN
NAGN
DAGN
DHTN
MHN

I-4B


GSH content (% of control)
Concentration (mM)
EAN
HDN
MAN

I-4C

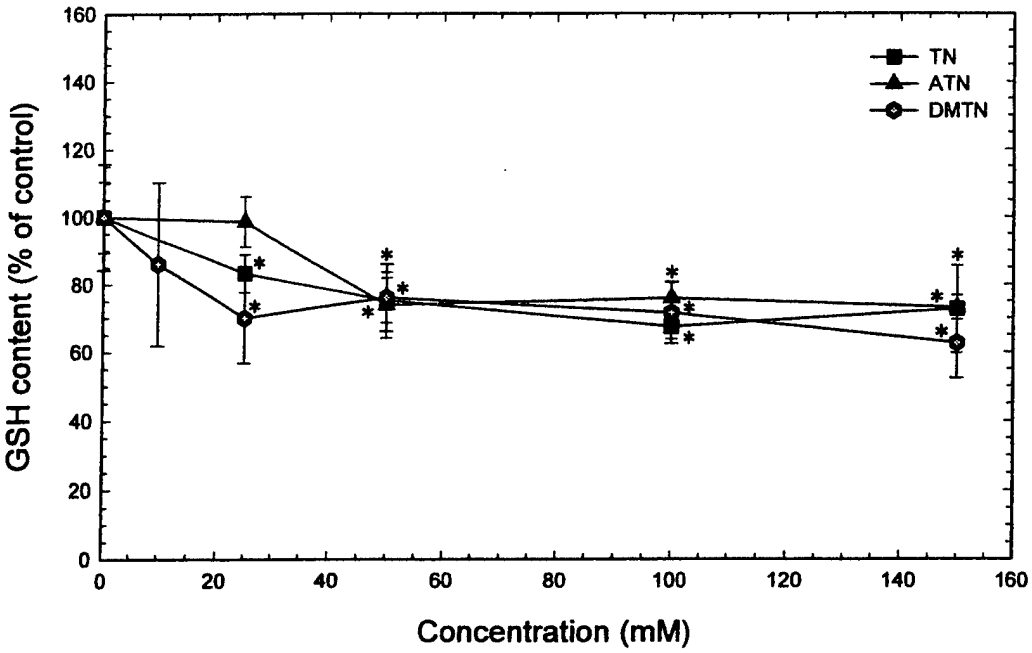
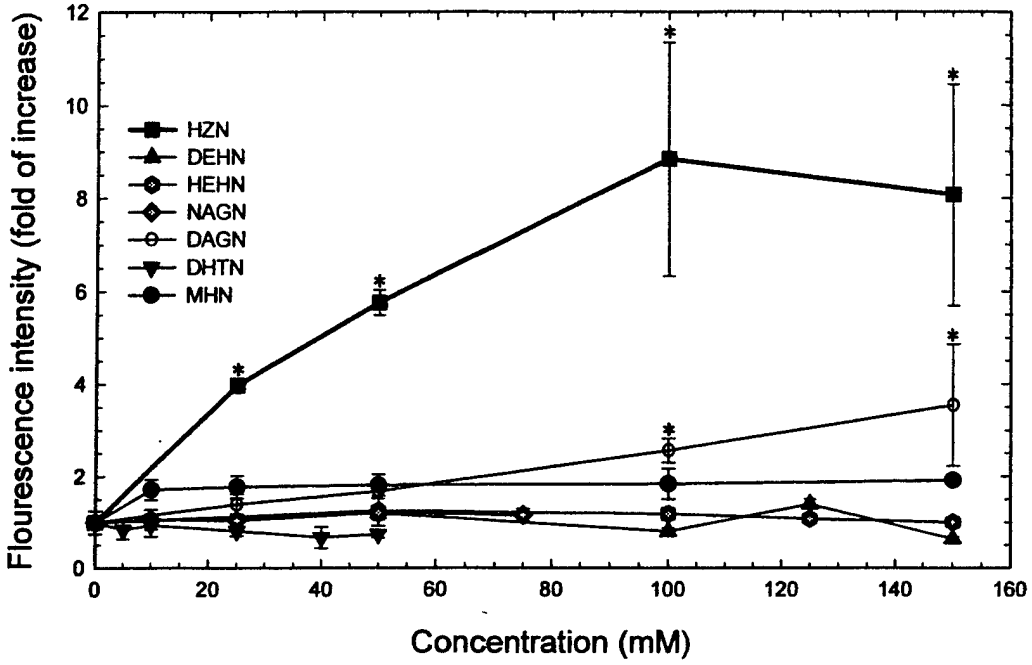


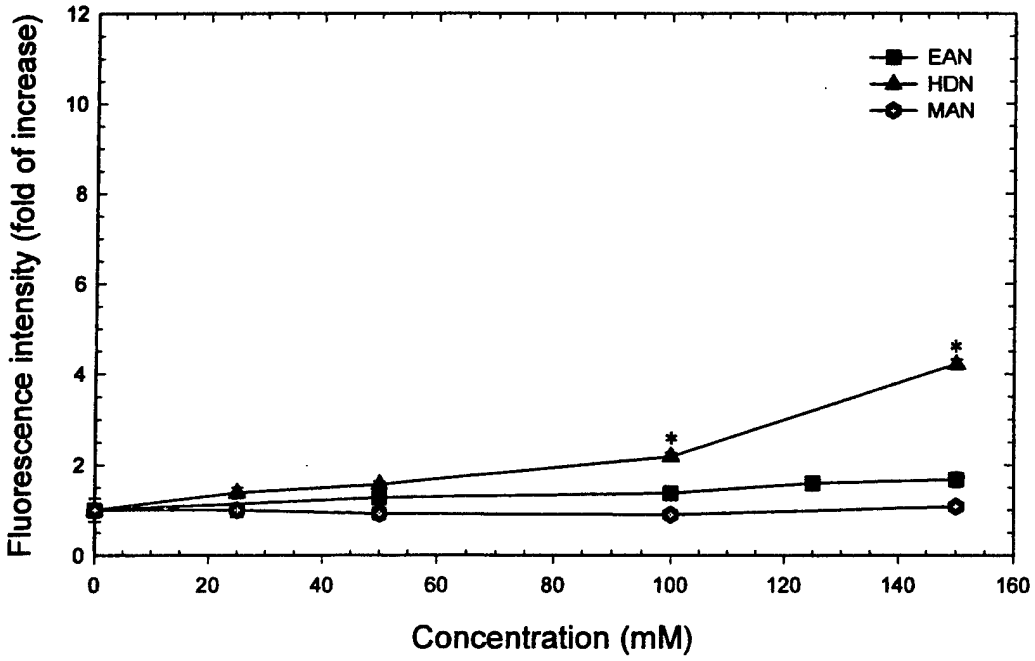
Figure I-4. Effect of HEC on GSH Levels in Hepatocytes.
GSH content is plotted as a function of HEC dose for hydrazine-based HEC in (A), for amine-based HEC in (B) and for triazole-based HEC (C). Primary hepatocytes were treated with different concentrations of HEC for 4 h. At the end of the exposure, cells were washed with PBS, and GSH levels were measured as described in the Materials and Methods section. The data are expressed as means ± SD of three independent experiments with hepatocytes from three different rats. (*) indicates a statistically significant difference compared to controls ($p < 0.05$).

Dichlorofluorescein diacetate (DCFH-DA) is widely used to measure reactive oxygen species (ROS) generation in cells. The ROS generation following exposure to HEC is shown in Figure I-5. Even a low dose of HZN (25 mM) produced a significant increase in ROS generation at the end of the 4-h exposure. The HZN treatment at 100 and 150 mM resulted in an approximately eight-fold increase in ROS. HZN is the most toxic followed by DAGN and MHN. Other hydrazine-containing compounds (HEHN, DEHN, NAGN, and DHTN) did not show significant increase in ROS. Among amino-containing compounds, HDN caused the greatest increase in ROS. An increase in ROS was observed in EAN-treated cells at higher dose (150 mM), although there was no significant effect observed in MAN-exposed cells. No appreciable increase in ROS generation was observed for cells treated with triazole-containing compounds (TN, ATN, and DMTN). Using ROS generation as a measure of toxicity, HZN was the most toxic of the HEC.

I-5A


Flourescence intensity (fold of increase)
Concentration (mM)
HZN
DEHN
HEHN
NAGN
DAGN
DHTN
MHN
0
2
4
6
8
10
12
0
20
40
60
80
100
120
140
160

I-5B


Fluorescence intensity (fold of increase)
Concentration (mM)
EAN
HDN
MAN
0
2
4
6
8
10
12
0
20
40
60
80
100
120
140
160

I-5C

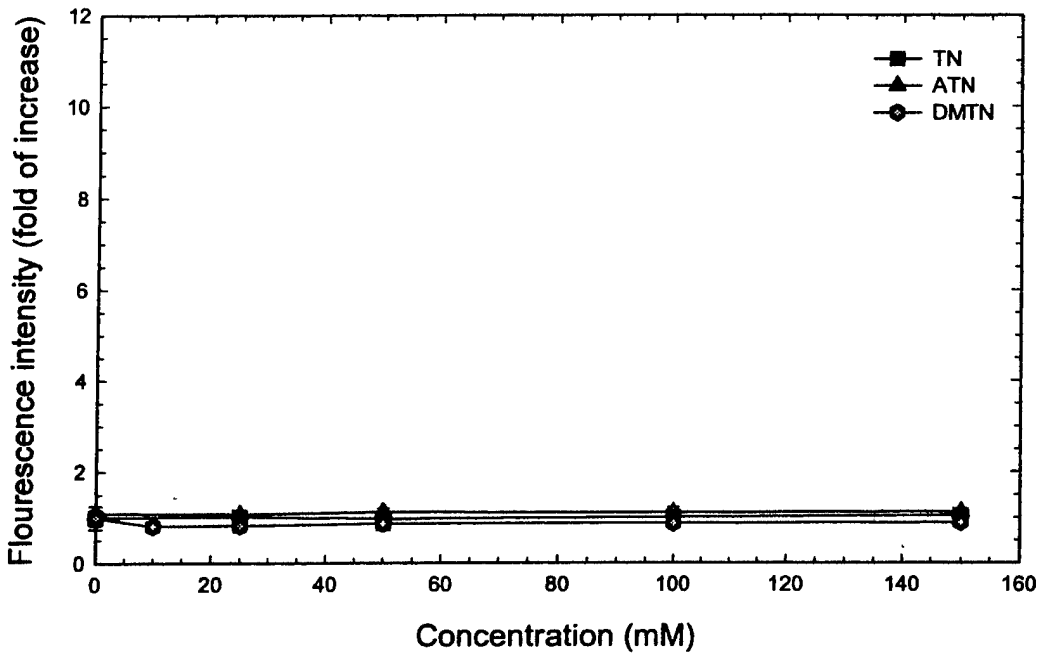


Figure I-5. Effect of HEC on ROS Generation in Hepatocytes.
The increase in ROS generation (fold of increase in fluorescence intensity) is plotted as a function of HEC dose for hydrazine-based HEC in (A), for amine-based HEC in (B) and for triazole-based HEC (C). Primary hepatocytes were incubated with DCFH-DA for 30 min. After DCFH-DA containing medium was removed, the cells were washed and treated with HEC in Chee media for 4 h. At the end of exposure, fluorescence was measured as described in Materials and Methods section and the intensity of fluorescence expressed in fold increase in treated cells with respect to control. The data are expressed as means ± SD of three independent experiments with hepatocytes from three different rats. (*) indicates a statistically significant difference compared to controls ($p < 0.05$).

Toxicity Comparison

In order to compare the results of the assays using values representative of a dose-response curve, the following parameters were determined: The lowest effective concentration (LEC) was determined for ROS and effective concentrations (EC) were determined for MTT, LDH, and GSH. The EC_{75} was determined in order to maximize the number of data points for MTT. Similarly, the EC_{25} was determined for LDH and the median effective concentration (EC_{50}) was determined for GSH. Many of these endpoints, displayed in Table I-2, can only be expressed as lower limits from the dose-response curves (greater than 150 mM).

TABLE I-2: CALCULATED EC_{75}, LOWEST EFFECTIVE CONCENTRATION (LEC), EC_{25} AND EC_{50} VALUES OF HEC

Propellants	MTT EC_{75} (mM)	ROS LEC (mM)	LDH EC_{25} (mM)	GSH EC_{50} (mM)
HZN	35	5	20	20
HEHN	68	>150	75	116
DEHN	50	>150	65	32
MHN	18	7	>150	58
DAGN	110	30	>150	145
EAN	115	105	30	144
HDN	58	25	>150	80
MAN	150	>150	>150	>150
TN	>150	>150	>150	>150
ATN	>150	>150	>150	>150
DMTN	>150	>150	>150	>150
DHTN	32	>150	30	4
NAGN	>150	>150	>150	>150

DISCUSSION

ROS are by-products of biological redox reactions and are involved in various pathological conditions (Farber *et al.*, 1990). A large increase in ROS results from a low dose of HZN among all HEC compounds. Increased generation of ROS by HEC is likely to contribute to oxidative stress that may ultimately manifest cytotoxicity. Increase in intracellular ROS, often referred to as oxidative stress, represents a potentially toxic insult, which, if not counteracted, will lead to membranous dysfunction, as well as protein and DNA damage (Preece and Timbrell 1989; Loft and, Poulsen 1999). The toxicity of HEC, revealed by LDH release and MTT reduction, is strongly correlated to ROS generation and indicates induction of massive oxidative stress *in vitro* in primary cultures of rat hepatocytes.

Glutathione is the principal intracellular non-protein thiol that is the major source of reducing power in the cell (Sies, 1999). It provides a primary defense against oxidative stress by its ability to scavenge free radicals. The results reported here show a dose-dependent depletion of GSH by HEC. It is interesting to note that low dose (25 mM) of HZN greatly depleted GSH compared to other HEC, which did not occur to any notable extent for either MTT reduction or LDH leakage. GSH depletion in primary culture of rat hepatocytes exposed to HEC is strongly correlated to the increased ROS generation. It is possible that GSH depletion makes cells produce reactive oxygen species. Previously, it has been shown the loss of GSH, an important cellular antioxidant, increased endogeneous ROS to toxic levels in hepatocytes (Anundi *et al.*, 1979). It has been postulated that the loss of GSH and catalase may compromise cellular antioxidant defenses and lead to the accumulation of ROS that are generated as by-products of normal cellular function (Hussain and Frazier 2001; Hussain *et al.*, 1999).

The major events in HEC cytotoxicity of primary rat hepatocytes are the reduction of mitochondrial function, generation of ROS and GSH depletion. It is not known the exact mechanism of action of these chemicals, but the depletion of GSH and generation of ROS strongly suggest that toxicity of HEC may be mediated through oxidative stress. This infers that the mechanism of biological response for MTT involves loss of an electron. But it must be remembered that the actual species used in the assays are the nitrate salts, which in solution, consist of the protonated form of the HEC and $[NO_3]^-$. Therefore, it is assumed that the biological mechanism of toxic response first involves loss of a proton to form the neutral HEC.

One of the aims of this study was to classify HEC based on mechanism of toxicity using an *in vitro* model. This would allow for the toxicity prediction of additional HEC proposed as new propellants or other applications and perhaps also provide insight into the biophysical mechanisms involved. The experimental results added to the hydrazine literature by demonstrating reduced mitochondrial function, increased LDH leakage, elevated ROS generation, and decreased GSH content at the end of 4-h exposures. There are number of reports available on hydrazine toxicity, however, there are no reports on toxicity of the HEC examined in this study. Hydrazine is known to deplete ATP, generate ROS and destabilize mitochondrial function (Kerai and Timbrell 1997). The results demonstrated that hydrazine-based compounds in general are more toxic than amine and triazole containing compounds. However, some chemicals displayed different toxicity; for example NAGN, although it belongs to hydrazine-based HEC, is a less toxic chemical. Similarly MAN which is an amine-based chemical and triazole-containing chemicals were relatively less toxic than other HEC based on the four toxicity end points tested. Based on these biochemical data, the chemicals were classified into three categories: higher toxicity (hydrazine containing compounds), medium toxicity (amino containing compounds), and lower toxicity (triazole containing compounds).

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IN VITRO RAT HEPATOCYTE TOXICITY AND BACTERIA GENOTOXICITY EVALUATION OF HIGH ENERGY CHEMICALS FOR REPLACEMENT OF HYDRAZINE

SECTION II. GENOTOXICITY ASSAYS FOR ELEVEN HIGH ENERGY COMPOUNDS: *SALMONELLA*/MICROSOME MUTAGENESIS ASSAY

Prepared by Sheela Sharma, Ph.D. and Pu Gao, M.D. (Cellular and Molecular Toxicology Program, Life Sciences and Toxicology Division, ManTech Environmental Technology, Inc., Research Triangle Park, NC), excerpted from Study No. 140-1126.

SUMMARY

The potential genotoxic effects of eleven high energy chemicals, HZN, DEHN, DHTN, DAGN, NAGN, EAN, HDN, MAN, TN, ATN and DMTN in *Salmonella*/Microsome mutagenicity assay (Ames Test) were investigated. In this assay, five bacterial strains (TA98, TA100, TA102, TA1535 and TA1537) were tested with and without the addition of activation system (S9). A modification of the standard plate incorporation method, the pre-incubation method was used to increase the sensitivity of the assay. The data from the range finding (dose selection) assay indicated that the concentrations used in the mutagenicity assay varied between chemicals for different strains. For example, 0.03-3.0 mg/plate were used for HZN (0.32-30 mM), DHTN (0.15-15 mM), NAGN (0.16-16 mM), MAN (0.27-27 mM) and DMTN (0.22-22mM); and 0.1-5.0 mg/plate were used for EAN (0.8-40 mM), HDN (0.42-21 mM), TN (0.75-38 mM) and ATN (0.67-34 mM), for all five strains. The concentrations for DAGN ranged from 0.03-3.0 mg/plate (0.2-2 mM) for tester strains TA100, TA102 and TA1535; and 0.1-5.0 mg/plate (0.54-32 mM) for strains TA98 and TA1537. Only range finding assay was performed for DEHN using tester strains TA98, TA100 and TA102, as there was a limited amount of chemical.

Results from the mutagenicity assay showed that, compared to the solvent control, HZN increased the revertants in TA102 and TA1535 in a dose-dependent manner at all concentrations tested (1.2-1.4 fold and 2.0-5.0 fold, respectively) in the presence of S9 activation system ($p<0.05$). DHTN increased the revertants in three tester strains (TA98, TA100 and TA102) with and without S9 activation system at concentrations of 0.03-0.3 mg/plate (0.15-15 mM) ($p<0.05$). Toxicity was observed at the highest concentrations (1 and 3 mg/plate or 5 and 15 mM) in all strains with significant increase in toxicity without S9. Unlike the effect in strains TA98 and TA102, DHTN showed a significant dose-dependent increase of revertants in TA100 in the presence of S9 (~2.0-4.0 fold). Similarly, in TA1535, DHTN also induced the mutant revertants in a dose-dependent manner at concentrations of 0.03-0.3 mg/plate (0.15-1.5 mM) with S9 (2.0-5.0 fold) and 0.03 and 0.1 mg/plate (0.15 and 0.5 mM) without S9 (~2 fold). MAN significantly increased (~5 fold) the revertants (non dose-dependent) in one tester strain (TA100) at all concentrations tested from 0.03-3.0 mg/plate (0.15-1.5 mM) with and without S9 activation system ($p<0.01$). HDN and DMTN showed a moderate but statistically significant increase in the

revertants only in tester strain TA102 at all concentrations with and without S9 ($p<0.05$). The concentrations of HDN that induced mutant revertants were 3.0-5.0 mg/plate with S9 and only 5.0 mg/plate (21 mM) without S9. The inducing concentrations for DMTN were 0.03-3.0 mg/plate (0.22-2.2 mM) with and without S9 activation system ($p<0.05$). The rest of the compounds (DAGN, NAGN, EAN, TN and ATN) were negative in this assay.

The data from this study indicated that HZN, DHTN and MAN are mutagens in the bacterial system by causing both base pair substitutions (GC for TA100, AT for TA102) and frameshift (TA98) mutations. DHTN is a direct mutagen as there was no statistically significant increase in the number of histidine revertants in the activated (+S9) group compared to the non-activated (-S9) group in all three strains ($p>0.05$). However, HZN and MAN can be considered as indirect mutagens, as there is a statistical difference in the number of histidine revertants between activated (+S9) group and non-activated (-S9) group ($p<0.05$). Both HDN and DMTN appear to be weak mutagens in the bacterial system, although it showed statistically significant induction of revertants, however, the increase in revertant numbers did not reach the standard criteria of 2 fold increase.

INTRODUCTION

The overall objective of the study is to determine the potential genotoxicity associated with the exposure to eleven high energy chemicals (HZN, DEHN, DHTN, DAGN, NAGN EAN, HDN, MAN, TN, ATN and DMTN), a new series of primarily hydrazine derivatives and amino-containing compounds with potential application as aircraft fuel and propellant to replace hydrazine, in *Salmonella*/Microsome mutagenicity assay (Ames Test). In a previous report, hydroxyethylhydrazinium nitrate (HEHN) elicited a positive mutagenic response in *Salmonella* strains TA102 and TA1535, but was negative in strains TA98, TA100, and TA1537 (Sharma and Gao, 1999). The *Salmonella*/mammalian microsome revertant mutation system is a well-defined short-term assay for the detection of carcinogens/mutagens. It measures the reversion from his^- (histidine dependent) to his^+ (histidine independent) induced by chemicals that cause base changes or frameshift mutations in the genome of the organism. In this assay, bacteria are exposed to the test agent with and without a metabolic activation system (Aroclor 1254 induced rat liver S9 with co-factors) and plated onto minimal agar medium that is deficient in histidine. After incubation for 48 hours, revertant colonies are counted and compared with the number of spontaneous revertants in vehicle control culture. The mutagenicity of test agents is evident by the increase of revertants. All assays were conducted in accordance with the provision of the United States Environmental Protection Agency/Toxic Substances Control Acts (EPA/TSCA) Good Laboratory Practice (GLP) standards as defined in the Federal Register (40 CFR Part 792, 2002), the EPA/TSCA Health Effect Testing Guideline (40 CFR 798.5265, 2001-2002) and EPA Health Effects Test Guideline (OPPTS 870. 5100, 1998). All the procedures were performed in accordance with the Standard Operating Procedures (SOPs) of the Cellular and Molecular Toxicology Program at ManTech Environmental.

METHODS

Materials

Salmonella typhimurium strains

Five tester strains (TA98, TA100, TA102, TA1535 and TA1537) were obtained from Dr. Bruce N. Ames, Deptartment of Molecular and Cell Biology, University of California at Berkeley (Ames *et al.*, 1975; Maron and Ames, 1983), stored at -80°C and used in this assay.

Metabolic activation system

Aroclor 1254-induced rat (Sprague-Dawley adult male) liver *S9 homogenate* (Cat # 11-101, Lot #1253 and 1289) was purchased from Moltox (Boone, N.C.), and stored at -80°C. It was diluted with cofactors to make the standard S9 activation mixture. The S9 mixture contains 33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose-6-phosphate, 4 mM NADP and 100 mM phosphate buffer (pH 7.4) and S9 (0.04 mL/mL mixture). The concentration (volume) of S9 is based on the historical data from the laboratory and the revised methods for the *Salmonella* mutagenicity test by Maron and Ames (1983). The S9 mixture was made fresh prior to use and kept on ice.

Growth medium

Bacto nutrient broth (Difco Laboratories, Cat # 0003-17-8, Lot # 116525JD) was prepared by dissolving 8 g powder and 5 g NaCl in 1 L of distilled water, which was sterilized and used for growing tester strains. The growth medium was routinely stored at 4°C.

Top agar

Top agar contains 0.6% Bacto agar (Difco Laboratory, Cat # 0140-05, Control # 653470,781733 and 1274000) and 0.5 % NaCl in distilled water, which was autoclaved and stored at room temperature. Before plating, 10 mL of sterile 0.5 mM histidine/0.5 mM biotin solution was added to 100 mL of melted top agar, kept at 45°C and used as an overlay on the minimal agar plate.

Minimal agar plate

The minimal agar was prepared by dissolving 1.5% Bacto agar (Difco Laboratory, Cat # 0140-05, Control # 715860) and 2% glucose in Vogel-Bonner medium E. Minimal agar plates were made by adding 30 mL of the minimal glucose agar medium onto a 100-mm x 15-mm bacterial plate. Vogel-Bonner medium E was prepared by dissolving 0.04 M $MgSO_4$, 0.52 M citric acid, 2.87 M K_2HPO_4, and 0.87 M $NaHNH_4$ in distilled water and sterilized. It was stored at 4°C.

Chemicals for genotypes confirmation

Crystal violet (Fisher, Cat # C581, Lot # 870757): 0.1% dissolved in distilled water.

Histidine (Sigma, Cat # H-8125, Lot # 63H0202): 0.1 M dissolved in distilled water and sterilized.

Biotin (Sigma, Cat # B-4501, Lot # 34H0932): 0.5 M dissolved in distilled water and sterilized.

Ampicillin (Sigma, Cat # A-9518, Lot # 85H0372): 8 mg/mL dissolved in 0.02N NaOH.

Tetracycline (Sigma, Cat # T3383, Lot # 43H1092): 8 mg / mL dissolved in 0.02N HCl.

Positive control chemicals

2-Anthramine (Sigma, Cat # A1381, CAS # 613-13-8, Lot 3 77H1867): dissolved in DMSO, further diluted with DDH_2O to 25 µg/mL and 2.5 µg/plate was used for all five tester strains with S9 metabolic activation system.

9-Aminoacridine (Sigma, Cat # A-7295, CAS # 90-45-9, Lot # 106FO6681): dissolved in DMSO and further diluted with DDH_2O to 500 μg/mL and 50 μg/plate was used for tester strain TA1537 without S9 metabolic activation system.

Mitomycin C (Sigma, Cat # M0503, CAS # 50-07-7, Lot # 71F-0634): dissolved in DMSO, further diluted with DDH_2O to 5 μg/mL and 0.5 μg/plate was used for tester strain TA102 without S9 metabolic activation system.

2- Nitrofluorene (Aldrich, Cat # N 1,675-4, CAS # 607-57-8, Lot # ES02408LR): dissolved in DMSO and further diluted with DDH_2O to 100 μg/mL, and 10 μg/plate was used for tester strain TA98 without S9 metabolic activation system.

Sodium azide (Sigma, Cat # S-2002, CAS # 26628-22-8, Lot # 113H0265): dissolved in DMSO and further diluted with DDH_2O to 20 μg/mL, and 2 μg/plate was used for tester strains TA100 and TA1535 without S9 metabolic activation system.

Test agents

Test chemicals were directly obtained from Air Force Research Lab, Edwards Air Force Base, CA. HZN appeared as white solid particulate, DEHN, DAGN, NAGN, HDN, MAN, TA, ATN and DMTN appeared as white crystalline solid; DHTN appeared as amber-red crystalline solid and EAN appeared as colorless to white crystalline solid. The chemicals were kept at -80°C, pre-weighed in a glove–box for safety and dissolved in DMSO prior to use.

Procedures

Culturing of tester strains

The tester strains, frozen at -80°C were thawed, inoculated in nutrient broth and incubated in an environmental shaker incubator at 37°C for12~15 hours to give the bacterial density of 1-2 x 10^9/mL. The bacteria were kept in a refrigerator prior to use.

Genotype confirmation

Genotypes of each strain were confirmed prior to the mutagenesis study, which included the requirement of histidine (His^-), the sensitivity to crystal violet (rfa mutation) and U.V. light (uvrB mutation), the resistance to ampicillin and tetracycline (R factor), ampicillin plus tetracycline for TA102 and ampicillin alone for the rest of four tester strains and the occurrence of spontaneous revertants.

Range-Finding Assay (Dose Selection)

A preliminary range-finding assay was performed using TA98, TA100 and TA102 or TA100 and TA102 to determine the optimal test concentrations for the mutagenesis assay. Five log concentrations (0.0005-5.0 mg/plate) of five chemicals (HZN, DEHN, DHTN, DAGN and EAN) were tested in TA98, TA100 and TA102; six (NAGN, HDN, MAN, TN, ATN and DMTN) were tested using TA100 and TA102 with modified standard plate incorporation, the pre-incubation method. All chemicals were dissolved in DMSO followed by four-log dilutions in DDH_2O, and no precipitation was observed in any of the chemical dilutions.

Mutagenesis Assay

Modified Standard Plate incorporation (pre-incubation method): In the mutagenesis assay, all chemicals were freshly dissolved in DMSO followed by four half log dilutions with DDH_2O prior to use, and no precipitation was observed.

For HZN, DHTN, NAGN, MAN and DMTN, 0.03-3.0 mg/plate were used in all five tester strains. For EAN, HDN, TN and ATN, 0.1-5.0 mg/plate were used in all five tester strains. For DAGN, the concentrations ranged from 0.03–3.0 mg/plate for tester strains TA100, TA102 and TA1535 and 0.1-5.0 mg/plate for tester strains TA98 and TA1537. Due to the limited amount of chemical, DEHN was tested only in the range-finding assay using tester strains TA98, TA100 and TA102.

One-tenth mL bacteria, 0.1 mL test agent and 0.5 mL S9 mixture (+S9 group) or 0.2 M phosphate buffer (-S9 group) were pre-incubated at 37°C for 20 min with shaking before 2 mL of top agar was added to this mixture. The contents were mixed, then poured onto the surface of a minimal glucose agar plate and spread out evenly. After the top agar was solidified, the plates were inverted and incubated at 37°C for 48 hours. The number of revertants per dish was counted by an automatic colony counter (AccuCount 1000, Biologics). The appearance of background lawn of bacterial growth was checked. Cultures were set up in triplicate; negative controls (spontaneous and solvent (DMSO) control) and positive controls were also included.

RESULTS

The raw data for the Ames Test are attached as Appendix A and the salient results are summarized as follows.

Genotype Identification

Different genotypes of the tester strains were verified by the standard procedure of B.N. Ames prior to the study. Results (see Table II-1) indicated that all the tester strains were qualified for the study.

TABLE II-1: GENOTYPE CONFIRMATION OF TESTER STRAINS

Genotypes	TA98	TA100	TA102	TA1535	TA1537
Histidine requirement	+	+	+	+	+
rfa mutation	+	+	+	+	+
uvrB mutation	+	+	–	+	+
R factor	+	+	+	–	–
Spontaneous Revertants	60 ± 3.8	150 ± 3.2	391 ± 11.7	17 ± 4.5	13 ± 4.7

Dose Selection for High Energy Chemicals

The conversion of mg/plate to mM concentration for each chemical in the dose selection assay is shown in Table II-2. The results from dose selection studies are listed in Tables II-3 through II-12. Based on the reduction compared to DMSO control revertants, toxicity was observed at 5 mg/plate for HZN, DHTN and DAGN in TA98, TA100 and TA102 and for NAGN in TA100 and TA102. Toxicity was noticed for MAN in TA100 at 5 mg/plate and slight toxicity in TA102 from 0.0005 to 0.5 mg/plate. TN showed a different toxicity pattern; 5 mg/plate was toxic only in S9-system in both TA100 and TA102. Similar toxicity pattern was noticed with DMTN in tester strain TA100 (toxicity only in the absence of S9 system). EAN and ATN were less toxic than the rest of the chemicals; either there was no toxicity or slight toxicity at all concentrations tested in all the tester strains. However, even when there was slight toxicity, no clearing of background lawn (indicating toxicity) was observed.

TABLE II-2: CONVERSION OF mg/PLATE TO mM CONCENTRATION FOR EACH CHEMICAL IN THE DOSE SELECTION ASSAY

Chemical	MW	Dose selection (log dose)	
		mg/plate	mM
HZN	95.05	0.0005-5	0.0050-50
DEHN	151.16	0.0005-5	0.0030-30
DHTN	205.13	0.0005-5	0.0024-24
DAGN	152.11	0.0005-5	0.0032-32
NAGN	182.09	0.0005-5	0.0027-27
EAN	124.09	0.0005-5	0.0040-40
HDN	237.17	0.0005-5	0.0021-21
MAN	110.07	0.0005-5	0.0045-45
TN	132.08	0.0005-5	0.0037-37
ATN	147.09	0.0005-5	0.0033-33
DMTN	138.12	0.0005-5	0.0036-36

TABLE II-3: RESULTS OF DOSE SELECTION ASSAYS FOR HZN

Treatment	TA98		TA100		TA102	
	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	37.89 ± 6.47	27.11 ± 6.54	141.22 ± 3.29	138.33 ± 8.50	350.78 ± 42.47	340.22 ± 23.31
DMSO	34.56 ± 6.27	27.89 ± 6.47	140.44 ± 15.07	137.78 ± 7.41	328.78 ± 31.65	358.22 ± 49.30
Anthramine	832.89 ± 160.84		1285.33 ± 103.94		518.78 ± 41.10	
Mitomycin C						1592.78 ± 25.47
2-Nitrofluorene		151.22 ± 21.00				
Sodium azide				501.33 ± 31.97		
HZN (mg / plate)						
0.0005	40.78 ± 7.03	26.33 ± 6.17	157.22 ± 23.80	145.00 ± 11.79	417.22 ± 30.97	322.78 ± 23.05
0.005	39.22 ± 4.54	43.56 ± 11.03	133.67 ± 30.56	134.89 ± 8.01	539.44 ± 30.20	313.89 ± 15.94
0.05	29.89 ± 8.83	24.22 ± 5.50	141.78 ± 21.19	123.22 ± 13.93	401.22 ± 28.66	349.78 ± 26.65
0.5	30.67 ± 4.33	37.22 ± 5.36	158.33 ± 18.68	150.67 ± 10.59	545.78 ± 5.68	333.22 ± 11.01
5	2.00 ± 0.33	0.00 ± 0.00	30.44 ± 18.21	0.00 ± 0.00	83.67 ± 41.76	0.00 ± 0.00

TABLE II-4. RESULTS OF DOSE SELECTION ASSAYS FOR DEHN

Treatment	TA98		TA100		TA102	
	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	28.67 ± 2.65	21.44 ± 0.69	89.33 ± 20.27	138.67 ± 21.84	252.00 ± 32.62	270.67 ± 34.04
DMSO	28.56 ± 2.01	18.11 ± 4.00	63.33 ± 14.77	120.33 ± 27.84	201.56 ± 14.63	244.22 ± 9.58
Anthramine	1476.44 ± 56.40		1362.56 ± 46.34		496.44 ± 45.73	
Mitomycin C						1342.67 ± 238.23
2-Nitrofluorene		482.11 ± 37.83				
Sodium azide				477.44 ± 77.30		
DEHN (mg / plate)						
0.0005	21.33 ± 4.67	26.78 ± 1.68	128.56 ± 29.41	135.33 ± 4.73	137.89 ± 46.74	163.33 ± 69.57
0.005	26.67 ± 2.52	27.11 ± 8.80	135.33 ± 39.70	103.00 ± 34.18	131.11 ± 8.37	221.67 ± 105.94
0.05	23.00 ± 2.91	23.22 ± 5.85	138.22 ± 33.01	119.33 ± 9.70	235.44 ± 64.95	229.00 ± 55.76
0.5	23.22 ± 3.10	17.89 ± 6.11	55.11 ± 15.83	84.56 ± 20.04	220.78 ± 82.10	300.89 ± 18.07
5	15.67 ± 3.21	24.11 ± 1.39	67.22 ± 19.17	105.67 ± 15.19	250.11 ± 31.67	271.67 ± 13.67

TABLE II-5: RESULTS OF DOSE SELECTION ASSAYS FOR DHTN

Treatment	TA98		TA100		TA102	
	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	43.67 ± 5.77	24.33 ± 1.45	178.89 ± 16.10	196.44 ± 25.70	314.78 ± 30.52	273.67 ± 17.69
DMSO	48.67 ± 16.84	28.11 ± 3.02	142.00 ± 15.76	175.56 ± 6.62	284.11 ± 12.95	246.44 ± 14.79
Anthramine	1980.89 ± 191.10		2202.44 ± 258.22		344.00 ± 29.81	
Mitomycin C						952.78 ± 96.27
2-Nitrofluorene		963.56 ± 60.75				
Sodium azide				597.56 ± 170.09		
DHTN (mg / plate)						
0.0005	27.78 ± 5.55	19.56 ± 4.30	106.11 ± 11.48	88.33 ± 9.68	318.78 ± 13.61	288.11 ± 66.55
0.005	16.44 ± 14.34	15.00 ± 12.99	115.33 ± 28.20	115.44 ± 20.57	408.89 ± 19.02	293.78 ± 25.82
0.05	44.44 ± 5.52	29.44 ± 4.35	157.11 ± 33.49	101.00 ± 50.02	434.78 ± 11.71	356.78 ± 7.24
0.5	18.67 ± 4.00	14.67 ± 3.48	0.00 ± 0.00	52.44 ± 90.84	509.89 ± 34.85	256.44 ± 14.38
5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

TABLE II-6: RESULTS OF DOSE SELECTION ASSAYS FOR DAGN

Treatment	TA98		TA100		TA102	
	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	43.67 ± 5.77	24.33 ± 1.45	178.89 ± 16.10	196.44± 25.70	314.78 ± 30.52	273.67 ± 17.69
DMSO	48.67 ± 16.84	28.11 ± 3.02	142.00 ± 15.76	175.56± 6.62	284.11 ± 12.95	246.44 ± 14.79
Anthramine	1980.89± 191.10		2202.44±258.22		344.00 ± 29.81	
Mitomycin C						952.78 ± 96.27
2-Nitrofluorene		963.56± 60.75				
Sodium azide				597.56±170.09		
DAGN (mg / plate)						
0.0005	20.89 ± 0.69	21.78 ± 0.84	128.89 ± 15.94	117.22± 11.72	310.56 ± 16.98	245.44 ± 51.09
0.005	29.33 ± 5.20	18.56 ± 6.26	128.33 ± 12.50	118.89± 8.23	291.33 ± 45.20	249.00 ± 4.67
0.05	39.00 ± 4.71	20.67 ± 3.84	87.78 ± 17.71	112.00± 7.33	286.44 ± 41.35	254.33 ± 27.65
0.5	16.67 ± 5.24	24.78 ± 8.70	131.22 ± 28.44	108.56± 1.95	296.89 ± 16.98	257.00 ± 19.86
5	9.00 ± 12.73	53.50 ± 39.36	0.00 ± 0.00	5.33 ± 9.24	197.44 ± 25.93	138.22 ± 12.06

TABLE II-7. RESULTS OF DOSE SELECTION ASSAYS FOR NAGN

Treatment	TA100		TA102	
	S9+	S9-	S9+	S9-
Spontaneous	202.78 ± 12.33	184.33 ± 5.00	314.78 ± 30.52	273.67 ± 17.69
DMSO	162.44 ± 18.09	161.67 ± 24.67	284.11 ± 12.95	246.44 ± 14.79
Anthramine	898.22 ± 73.24		344.00 ± 29.81	
Mitomycin C				952.78 ± 96.27
Sodium azide		525.33 ± 64.89		
NAGN (mg / plate)				
0.0005	177.56 ± 22.37	141.44 ± 17.02	320.56 ± 44.98	307.11 ± 8.57
0.005	179.22 ± 12.67	187.89 ± 7.83	365.78 ± 72.68	294.22 ± 8.49
0.05	205.11 ± 52.24	157.67 ± 8.54	311.33 ± 38.42	281.33 ± 13.04
0.5	147.11 ± 38.09	125.33 ± 15.07	295.22 ± 18.81	261.33 ± 16.19
5	61.56 ± 3.98	61.78 ± 42.47	110.67 ± 56.20	218.56 ± 326.87

TABLE II-8. RESULTS OF DOSE SELECTION ASSAYS FOR EAN

Treatment	TA98		TA100		TA102	
	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	28.67 ±2.65	21.44 ±0.69	145.56 ±31.89	125.00 ±5.78	308.56 ±16.27	311.00 ±16.52
DMSO	28.56 ±2.01	18.11 ±4.00	113.89 ±24.84	107.00 ±24.06	325.89 ±20.55	273.00 ±9.24
Anthramine	1476.44 ±56.40		310.44 ±58.22		471.33 ±68.31	
Mitomycin C						797.22 ±55.06
2-Nitrofluorene		482.11±37.83				
Sodium azide				576.44 ±81.08		
EAN (mg / plate)						
0.0005	28.44 ±10.36	17.44 ±7.40	107.78 ±38.74	104.67 ±9.71	261.56 ±17.23	170.44 ±13.00
0.005	31.11 ±14.55	22.00 ±4.10	309.44 ±25.67	211.33 ±11.02	95.22 ±22.07	112:33 ±4.93
0.05	76.89 ±12.19	18.56 ±2.36	352.44 ±33.61	227.33 ±10.17	104.33 ±8.14	112.67 ±14.11
0.5	47.56 ±5.87	22.78 ±7.65	325.67 ±5.93	212.11 ±14.34	93.78 ±26.88	101.56 ±4.34
5	74.56 ±8.07	19.67 ±3.38	349.89 ±16.55	252.11 ±34.58	85.89 ±32.24	94.11 ±15.64

TABLE II-9: RESULTS OF DOSE SELECTION ASSAYS FOR HDN

Treatment	TA100		TA102	
	S9+	S9-	S9+	S9-
Spontaneous	118.89 ± 37.54	116.56 ± 13.80	319.33 ± 33.53	251.33 ± 45.21
DMSO	159.11 ± 26.46	89.78 ± 20.46	298.44 ± 19.47	207.89 ± 22.31
Anthramine	539.33 ± 51.52		562.00 ± 21.50	
Mitomycin C				1095.67 ± 22.98
Sodium azide		364.67 ± 19.70		
HDN (mg / plate)				
0.0005	78.78 ± 6.94	91.22 ± 24.44	304.78 ± 52.86	237.78 ± 13.83
0.005	84.78 ± 36.85	85.33 ± 13.12	237.00 ± 53.70	232.78 ± 23.17
0.05	57.11 ± 20.77	134.33 ± 23.09	280.00 ± 26.10	276.67 ± 32.22
0.5	65.67 ± 13.62	77.44 ± 31.45	272.89 ± 16.69	237.78 ± 19.03
5	78.22 ± 17.72	83.22 ± 22.61	280.89 ± 64.36	270.56 ± 13.67

TABLE II-10: RESULTS OF DOSE SELECTION ASSAYS FOR MAN

Treatment	TA100		TA102	
	S9+	S9-	S9+	S9-
Spontaneous	120.89 ± 39.90	116.56 ± 13.80	319.33 ± 33.53	240.22 ± 41.00
DMSO	159.11 ± 26.46	89.78 ± 20.46	298.44 ± 19.47	207.89 ± 22.31
Anthramine	539.33 ± 51.52		562.00 ± 21.50	
Mitomycin C				1095.67 ± 22.98
Sodium azide		364.67 ± 19.70		
MAN (mg / plate)				
0.0005	50.56 ± 12.40	116.33 ± 18.41	233.00 ± 19.20	198.78 ± 11.03
0.005	97.78 ± 20.00	120.78 ± 21.49	237.00 ± 26.03	176.56 ± 11.13
0.05	91.78 ± 24.76	96.67 ± 30.55	226.78 ± 28.17	194.78 ± 30.16
0.5	127.67 ± 42.85	78.56 ± 8.49	217.11 ± 24.07	166.89 ± 24.05
5	7.89 ± 13.66	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

TABLE II-11: RESULTS OF DOSE SELECTION ASSAYS FOR TN

Treatment	TA100		TA102	
	S9+	S9-	S9+	S9-
Spontaneous	174.56 ± 28.25	160.33 ± 22.88	348.00 ± 11.50	287.78 ± 37.45
DMSO	150.89 ± 16.98	140.89 ± 8.57	254.22 ± 46.50	316.33 ± 38.48
Anthramine	1148.33 ± 201.68		430.67 ± 26.26	
Mitomycin C				985.22 ± 35.79
Sodium azide		670.00 ± 124.38		
TN (mg / plate)				
0.0005	125.67 ± 4.26	155.78 ± 30.68	409.00 ± 11.85	343.33 ± 38.68
0.005	166.89 ± 2.80	172.33 ± 10.26	381.33 ± 42.61	379.67 ± 34.40
0.05	175.56 ± 16.20	159.78 ± 3.47	379.22 ± 40.82	313.00 ± 12.90
0.5	140.22 ± 24.09	158.89 ± 13.38	416.56 ± 9.71	341.22 ± 27.68
5	93.44 ± 9.31	15.22 ± 20.35	283.67 ± 54.62	64.00 ± 17.94

TABLE II-12. RESULTS OF DOSE SELECTION ASSAYS FOR ATN

Treatment	TA100		TA102	
	S9+	S9-	S9+	S9-
Spontaneous	174.56 ± 28.25	160.33 ± 22.88	348.00 ± 11.50	287.78 ± 37.45
DMSO	150.89 ± 16.98	140.89 ± 8.57	254.22 ± 46.50	316.33 ± 38.48
Anthramine	1148.33 ± 201.68		430.67 ± 26.26	
Mitomycin C				985.22 ± 35.79
Sodium azide		670.00 ± 124.38		
ATN (mg / plate)				
0.0005	149.00 ± 15.53	172.67 ± 10.17	342.00 ± 27.02	344.22 ± 35.30
0.005	141.67 ± 24.29	173.67 ± 15.41	387.89 ± 39.17	348.33 ± 9.33
0.05	176.89 ± 14.72	150.44 ± 8.92	377.33 ± 26.41	337.00 ± 15.31
0.5	143.56 ± 13.59	158.89 ± 17.83	392.56 ± 22.62	324.22 ± 33.97
5	95.00 ± 22.21	116.78 ± 143.52	373.89 ± 35.19	252.33 ± 73.96

TABLE II-13. RESULTS OF DOSE SELECTION ASSAYS FOR DMTN

Treatment	TA100		TA102	
	S9+	S9-	S9+	S9-
Spontaneous	145.56 ± 31.89	125.00 ± 5.78	308.56 ± 16.27	311.00 ± 16.52
DMSO	113.89 ± 24.84	107.00 ± 24.06	325.89 ± 20.55	273.00 ± 9.24
Anthramine	310.44 ± 58.22		471.33 ± 68.31	
Mitomycin C				797.22 ± 55.06
Sodium azide		576.44 ± 81.08		
DMTN (mg / plate)				
0.0005	136.11 ± 87.81	92.33 ± 30.66	321.56 ± 18.63	206.00 ± 16.17
0.005	76.67 ± 2.03	108.44 ± 16.51	275.89 ± 28.57	232.44 ± 23.26
0.05	98.78 ± 12.36	112.89 ± 0.84	247.44 ± 24.65	222.56 ± 16.82
0.5	119.22 ± 50.80	88.00 ± 18.28	316.22 ± 11.33	243.00 ± 31.18
5	97.22 ± 25.38	0.00 ± 0.00	407.56 ± 8.85	307.44 ± 23.00

Mutagenicity Assay

The results of mutagenicity assay with five tester strains (TA98, TA100, TA102, TA1535, and TA1537) are summarized in Tables II-14 through II-23. The data are expressed as the average revertant number per plate from the triplicates. The results indicate that compared to the solvent control, HZN, DHTN, HDN, MAN, and DMTN increased revertant mutant numbers either at three, four or five concentrations.

HZN

HZN was tested from 0.03-3.0 mg/plate. At 3 mg/plate, toxicity was observed in TA98, TA100 and TA1537 with and without S9 system and in the case of TA102 and TA1535 without S9 activation system. Surprisingly, 1 mg/plate was toxic to all five strains without S9 activation system. HZN increased the mutant revertants in TA1535 in a dose-dependent manner with 2.0-5.0 fold induction in the presence of S9 activation system when compared to the DMSO control ($p<0.01$). HZN also increased the revertants in TA102 at concentrations of 0.1-1.0 mg/plate with S9 activation system ($p<0.05$). At 3 mg/plate, a slight decrease in revertant numbers was observed, probably because HZN was slightly toxic at this concentration. In addition, HZN revealed a toxic-related dose response in TA1535 in the absence of S9 activation system (Table II-14 and Figures II-1 and II-2).

DHTN

DHTN was tested at 0.03-3.0 mg/plate, and toxicity was observed at 1 and 3 mg/plate for all tester strains except TA102 with S9 system. DHTN increased the mutant revertants in four tester strains (TA98, TA100, TA102 and TA1535) with and without S9 activation system at concentrations of 0.03-0.3 mg/plate by 2.0-4.0 fold and 1.5-2.0 fold, respectively ($p<0.05$) (Table II-15 and Figures II-3 and II-4).

HDN

HDN was tested at 0.1-5.0 mg/plate in all five strains; there was no toxicity in any of the concentrations tested. HDN showed a moderate but statistically significant increase of mutant revertants in tester strain TA102 at 3 and 5 mg/plate in the presence of S9 and 5 mg/plate in the absence of S9 ($p<0.05$). However, the increase in revertant numbers did not reach the two fold induction criteria for this assay and is rated as a weak mutagen (Table II-16).

MAN

MAN was tested at 0.03-3.0 mg/plate, toxicity was observed at 3 mg/plate for tester strains TA98, TA102, TA1535 and TA1537 and there was no demonstrated induction of mutation. For tester strain TA100, there was toxicity in the absence of S9 system but not in the presence of S9 system at a concentration of 3 mg/plate. In the presence and absence of S9 system, there was a

highly significant increase of the mutant revertant numbers (non dose-dependent) at 0.03-3.0 mg/plate and 0.03-1.0 mg/plate by 4.4–4.6 fold and 3.3-4.5 fold, respectively ($p<0.01$) (Table II-17 and Figure II-5).

DMTN

DMTN was tested at 0.03-3.0 mg/plate; there was no toxicity at all concentrations tested. DMTN increased the mutant revertants at 0.03-3.0 mg/plate in TA102 in both with and without S9 system. The increase in revertant numbers compared to solvent control was 1.3-1.4 and 1.4-1.6 fold, respectively. Although the increase did not reach the criteria for this assay (see above for HDN), it was still statistically significant ($p<0.05$) and therefore DMTN can also be considered as a weak mutagen (Table II-18).

DAGN

DAGN was tested at 0.03-3.0 mg/plate for tester strains TA100, TA102 and TA1535, 0.1-5.0 mg/plate for TA98 and TA1537. There was toxicity at 3 mg/plate in TA100 and TA1535 without S9 system. Similarly, at 5 mg/plate, DAGN was toxic for TA98 without S9 system. There was no increase in mutant revertant numbers at any of the tested concentrations in all five strains (Table II-19).

NAGN

NAGN was tested at 0.03-3.0 mg/plate. Toxicity was seen at 3 mg/plate in TA100, TA102 and TA1535 without S9 system. The mutant revertant numbers were increased at 3 mg/plate in TA98 without S9 system. Since only one dose induced the mutation frequency, NAGN is classified as non mutagenic (Table II-20).

EAN

EAN was tested at 0.1-5.0 mg/plate. There was slight toxicity at 5 mg/plate for TA98 and TA100 with and without S9 system, and no mutagenicity observed at all tested concentrations in all five tester strains (Table II-21).

TN

TN was tested at 0.1-5.0 mg/plate. TN demonstrated toxicity at concentrations of 5 mg/plate for all tester strains in both with and without S9 system. At a concentration of 3 mg /plate, TN was toxic to all five strains without S9 system, and there was no increase in revertant numbers (Table II-22).

ATN

ATN was tested at concentrations of 0.1-5.0 mg/plate. At 5 mg/plate, toxicity was found in TA98 and TA1537 without S9 system; however, an increased number of revertants were observed with S9 system. In TA100, TA102, TA1535, and TA1537, ATN demonstrated toxicity in both systems at 5 mg/plate. At a concentration of 3 mg/plate, ATN was toxic to TA102 with S9 system and TA1535 without S9 system. A slight increase in the number of revertants was noticed in TA98 and TA1535 with S9 system and TA102 without S9 system. However, the induction of revertants did not show any dose response pattern or statistical significance ($p>0.05$) (Table II-23).

DEHN

Due to the limited amount of chemical, only dose selection assay was performed for DEHN using three tester strains, TA98, TA100 and TA102. The data showed that DEHN was slightly toxic in TA100 at a dose of 0.5 and 5 mg/plate (Table II-3).

The experimental summary of 11 high energy chemicals is presented in Table II-24 (please note that there is no mutagenicity data for DEHN).

TABLE II-14: MUTAGENICITY ASSAY RESULTS OF HZN

Treatment	TA98		TA100		TA102		TA1535		TA1537	
	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	21.89 ± 3.15	17.56 ± 0.51	113.67 ± 19.34	90.78 ± 10.94	188.33 ± 24.58	165.89 ± 15.88	8.00 ± 2.03	16.67 ± 2.33	10.89 ± 2.17	6.78 ± 1.71
DMSO	21.11 ± 1.39	19.78 ± 5.19	102.33 ± 9.94	114.00± 5.33	262.22± 8.03	233.67 ± 7.21	13.22 ± 2.27	12.00 ± 1.73	11.78 ± 1.26	8.89 ± 0.96
Anthramine	819.33 ± 31.15		1160.89± 122.88		650.89 ± 105.51		182.11 ± 11.74		206.44 ± 3.20	
Aminoacridine										128.89 ± 13.93
Mitomycin C						1313.11 ± 5.80				
2-Nitrofluorene		380.00 ± 29.02								
Sodium azide				497.33± 121.10				475.11 ± 24.05		
HZN (mg) (mM)										
0.03 0.32	27.33 ± 6.84	20.33 ± 5.77	125.33 ± 17.93	102.67± 8.35	255.44± 15.54	210.00 ± 14.15	28.78 ± 2.69	16.89 ± 3.67	8.67 ± 1.76	8.89 ± 1.26
0.1 1	26.00 ± 6.43	15.56 ± 3.36	124.22 ± 12.20	104.33± 5.21	289.89± 17.26	210.67 ± 11.55	45.11 ± 4.11	16.11 ± 3.34	9.56 ± 2.78	7.89 ± 3.02
0.3 3	27.67 ± 1.53	8.33 ± 2.33	132.56 ± 8.77	118.78± 12.21	323.89± 1.84	248.78 ± 24.41	60.22 ± 5.55	12.00 ± 2.65	8.44 ± 2.22	6.89 ± 0.84
1 10	20.67 ± 4.33	0.00 ± 0.00	136.56 ± 4.67	0.00 ± 0.00	347.11± 28.97	0.00 ± 0.00	76.67 ± 19.35	2.67 ± 1.15	11.56 ± 2.22	0.00 ± 0.00
3 30	8.78 ± 1.07	0.00 ± 0.00	46.78 ± 3.56	0.00 ± 0.00	206.67± 26.35	0.00 ± 0.00	40.33 ± 8.45	0.00 ± 0.00	6.11 ± 1.84	0.00 ± 0.00

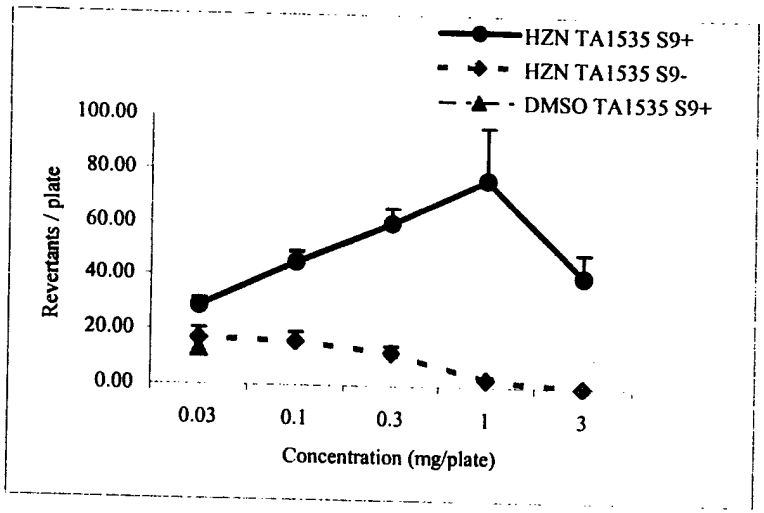

Figure II-1: Mutant Revertants Induced by HZN in TA1535 with and without S9 Activation System

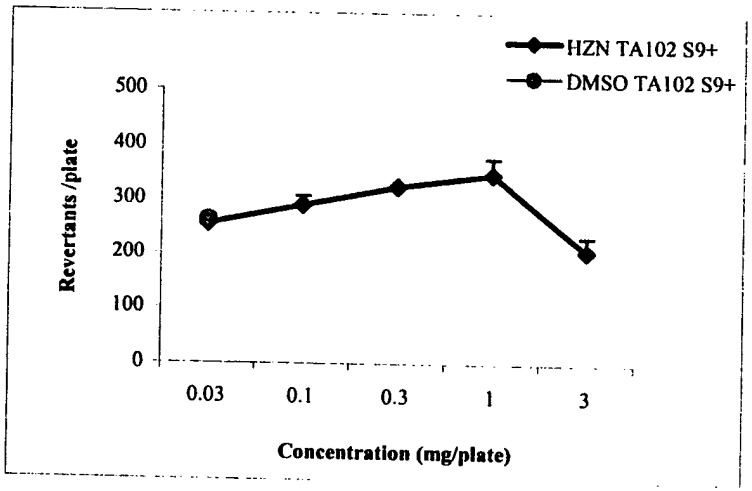

Figure II-2: Mutant Revertants Induced by HZN in TA102 with S9 Activation System

TABLE II-15: MUTAGENICITY ASSAY RESULTS OF DHTN

Treatment	TA98		TA100		TA102		TA1535		TA1537	
	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	27.67 ± 13.17	33.44 ± 11.41	147.22 ± 19.79	154.11 ± 9.65	343.78 ± 18.96	252.56 ± 53.53	12.33 ± 2.40	11.56 ± 7.90	11.33 ± 1.45	12.22 ± 0.77
DMSO	31.78 ± 10.46	25.33 ± 4.04	132.11 ± 7.50	116.11 ± 17.48	277.22 ± 53.93	236.78 ± 21.99	8.89 ± 1.50	10.78 ± 3.86	16.00 ± 4.26	3.36 ± 3.36
Anthramine	1090.56 ± 155.50		1780.00 ± 229.90		619.78 ± 69.65		193.22 ± 25.60		325.78 ± 17.83	
Aminoacridine										164.33 ± 53.26
Mitomycin C						905.89 ± 69.28				
2-Nitrofluorene		484.67 ± 21.30								
Sodium azide				702.67 ± 38.28				530.22 ± 7.89		
DHTN (mg) (mM)										
0.03 0.15	66.56 ± 7.90	52.44 ± 11.18	251.11 ± 41.96	252.33 ± 20.58	425.44 ± 54.72	425.22 ± 26.10	18.56 ± 5.82	17.67 ± 3.93	13.78 ± 1.71	12.00 ± 3.84
0.1 0.5	52.89 ± 5.52	69.33 ± 3.84	447.33 ± 82.67	401.89 ± 84.96	517.56 ± 35.83	436.56 ± 22.04	28.78 ± 11.46	21.78 ± 6.52	13.67 ± 0.67	13.11 ± 4.17
0.3 1.5	51.78 ± 15.87	46.78 ± 4.34	488.78 ± 78.53	212.11 ± 39.12	495.22 ± 21.55	421.67 ± 41.06	43.56 ± 4.95	8.33 ± 3.48	13.67 ± 4.81	8.11 ± 1.64
1 5	15.56 ± 13.73	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	427.67 ± 12.86	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	15.22 ± 4.48	0.00 ± 0.00
3 15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

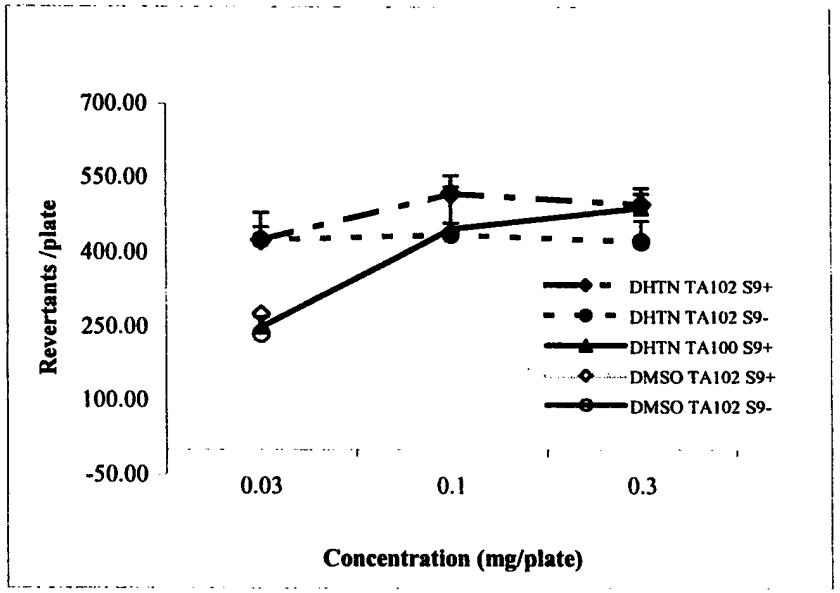

Figure II-3: Mutant Revertants Induced by DHTN in TA102 with and without S9 Activation System

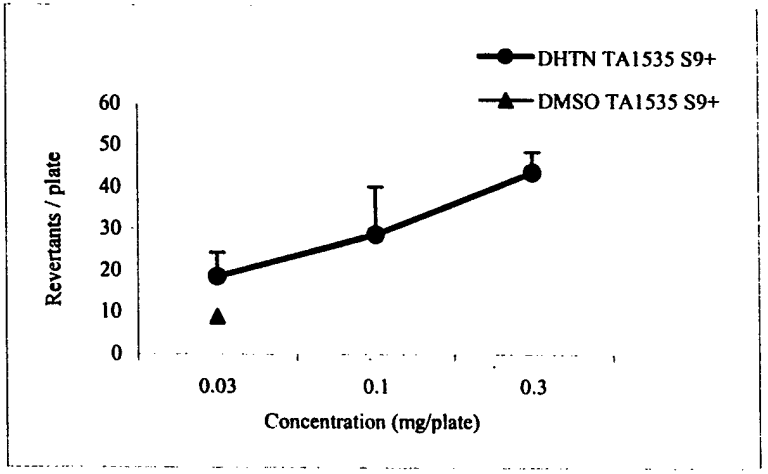

Figure II-4: Mutant Revertants Induced by DHTN in TA1535 with and without S9 Activation System

TABLE II-16: MUTAGENICITY ASSAY RESULTS OF HDN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		58.56 ± 9.89	43.78 ± 2.14	153.00 ± 18.48	162.22 ± 10.08	356.78 ± 47.00	273.22 ± 24.62	13.22 ± 1.02	16.33 ± 1.67	10.33 ± 1.15	13.78 ± 4.17
DMSO		54.89 ± 8.04	38.33 ± 3.76	140.44 ± 34.00	156.89 ± 13.81	301.11 ± 24.69	260.33 ± 24.95	14.00 ± 1.20	16.11 ± 3.79	12.56 ± 5.05	13.33 ± 2.08
Anthramine		1293.89 ± 299.46		1414.00 ± 169.75		550.11 ± 83.05		126.44 ± 8.13		104.67 ± 3.48	
Aminoacridine											109.00 ± 45.71
Mitomycin C							1112.11 ± 28.47				
2-Nitrofluorene			473.89 ± 23.82								
Sodium azide					654.33 ± 27.50				595.44 ± 32.36		
HDN (mg)	(mM)										
0.1	0.42	50.56 ± 3.67	39.44 ± 6.19	155.33 ± 3.28	143.33 ± 10.48	340.78 ± 11.80	269.00 ± 43.18	17.89 ± 3.40	15.67 ± 4.91	14.78 ± 6.19	10.89 ± 0.69
0.3	1.2	50.78 ± 8.32	35.33 ± 4.93	152.56 ± 19.25	151.78 ± 10.87	330.44 ± 63.84	290.56 ± 16.55	16.11 ± 6.54	14.22 ± 1.07	13.11 ± 5.17	11.89 ± 3.42
1	4.2	39.44 ± 5.17	34.33 ± 2.60	151.22 ± 12.51	150.89 ± 11.67	321.44 ± 49.38	258.56 ± 16.84	15.33 ± 0.33	12.78 ± 6.19	14.67 ± 4.51	10.44 ± 1.26
3	12.6	41.67 ± 4.16	45.11 ± 7.62	162.44 ± 15.33	162.22 ± 7.46	384.44 ± 16.58	267.33 ± 4.16	9.22 ± 1.02	14.22 ± 1.84	14.33 ± 0.33	12.78 ± 4.53
5	21	42.56 ± 6.50	36.33 ± 2.40	170.56 ± 14.38	161.00 ± 8.51	501.33 ± 12.55	398.00 ± 19.94	15.44 ± 4.35	12.67 ± 3.71	17.56 ± 2.41	9.00 ± 0.88

TABLE II-17: MUTAGENICITY ASSAY RESULTS OF MAN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		38.11 ± 1.84	31.11 ± 2.99	107.11 ± 12.76	159.78 ± 38.72	323.44 ± 21.18	318.89 ± 10.99	15.33 ± 6.17	11.67 ± 4.06	14.22 ± 5.23	14.22 ± 3.24
DMSO		38.22 ± 10.69	31.44 ± 8.44	275.56 ± 33.76	241.78 ± 39.08	285.44 ± 29.25	246.67 ± 19.64	9.44 ± 1.17	11.00 ± 4.26	14.67 ± 5.29	10.56 ± 2.67
Anthramine		398.44 ± 140.99		1260.00 ± 90.99		389.22 ± 19.62		105.00 ± 23.41		147.67 ± 18.50	
Aminoacridine											136.67 ± 43.92
Mitomycin C							926.11 ± 61.48				
2-Nitrofluorene			347.33 ± 20.22								
Sodium azide					944.33 ± 54.25				579.78 ± 47.89		
MAN (mg)	(mM)										
0.03	0.27	35.11 ± 7.76	32.00 ± 4.04	1279.89 ± 43.66	1047.78 ± 89.30	294.00 ± 91.00	269.00 ± 42.15	14.56 ± 2.12	9.56 ± 1.92	11.56 ± 4.07	8.89 ± 2.67
0.1	0.9	50.22 ± 12.22	42.78 ± 5.23	1226.22 ± 54.18	1047.56 ± 93.08	341.89 ± 3.56	249.00 ± 17.44	10.22 ± 2.71	7.33 ± 2.19	12.00 ± 1.00	10.56 ± 5.50
0.3	2.7	26.67 ± 5.49	29.78 ± 2.27	1249.44 ± 23.57	1087.67 ± 33.71	314.00 ± 21.11	286.33 ± 15.18	13.44 ± 7.49	13.56 ± 4.35	10.33 ± 2.85	10.44 ± 3.56
1	9	28.33 ± 3.79	24.22 ± 3.24	1278.78 ± 23.87	797.44 ± 32.15	268.89 ± 12.54	204.89 ± 26.70	22.78 ± 11.67	20.00 ± 6.23	6.33 ± 3.06	5.00 ± 4.84
3	27	2.67 ± 2.67	0.00 ± 0.00	1213.33 ± 15.70	32.67 ± 29.42	0.00 ± 0.00	0.00 ± 0.00	6.00 ± 3.76	0.00 ± 0.00	1.56 ± 1.39	2.11 ± 3.66

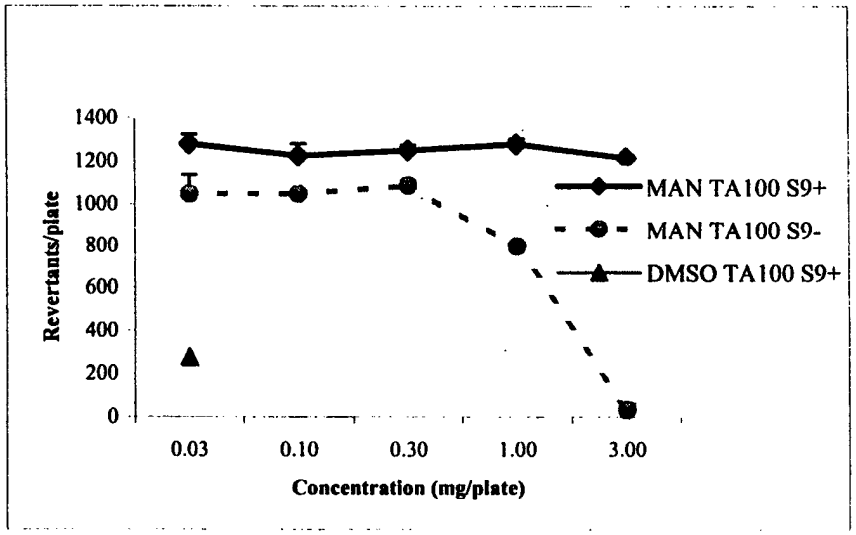

Figure II-5: Mutant Revertants Induced by MAN in TA100 with and without S9 Activation System

TABLE II-18: MUTAGENICITY ASSAY RESULTS OF DMTN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		31.56 ± 0.96	32.00 ± 2.08	153.00 ± 18.48	162.22 ± 10.08	220.67 ± 16.38	189.33 ± 15.68	18.89 ± 7.24	14.22 ± 1.07	8.33 ± 3.18	10.33 ± 1.86
DMSO		26.78 ± 1.71	22.11 ± 0.84	140.44 ± 34.00	156.89 ± 13.81	268.78 ± 30.93	197.11 ± 36.72	16.67 ± 4.62	11.33 ± 2.33	11.89 ± 1.58	8.67 ± 2.33
Anthramine		868.00 ± 77.66		1414.00 ± 169.75		273.89 ± 20.60		104.56 ± 28.59		76.78 ± 25.71	
Aminoacridine											107.33 ± 32.52
Mitomycin C							895.00 ± 58.86				
2-Nitrofluorene			586.67 ± 66.73								
Sodium azide					654.33 ± 27.50				188.67 ± 20.33		
DMTN (mg)	(mM)										
0.03	0.22	40.00 ± 9.26	37.44 ± 6.84	149.11 ± 3.15	164.00 ± 9.50	348.56 ± 60.63	308.00 ± 6.33	20.56 ± 8.00	15.44 ± 3.56	15.67 ± 8.33	11.44 ± 3.34
0.1	0.7	35.67 ± 7.69	46.78 ± 4.67	171.44 ± 10.03	151.33 ± 15.28	356.33 ± 24.67	284.78 ± 25.93	14.33 ± 3.93	13.00 ± 1.76	11.67 ± 5.33	10.33 ± 1.53
0.3	2.2	31.22 ± 2.67	43.00 ± 5.78	168.44 ± 13.50	155.22 ± 9.70	354.11 ± 24.35	291.56 ± 4.03	11.44 ± 3.98	13.11 ± 3.37	10.00 ± 1.20	11.44 ± 3.36
1	7	43.67 ± 5.20	50.22 ± 6.08	152.56 ± 7.24	157.22 ± 15.72	349.78 ± 57.58	294.33 ± 24.44	16.22 ± 4.74	11.11 ± 1.26	8.22 ± 0.96	10.22 ± 0.38
3	22	41.44 ± 4.67	39.67 ± 5.24	193.44 ± 10.51	109.00 ± 4.91	381.33 ± 52.85	332.33 ± 19.34	11.00 ± 3.93	9.67 ± 3.53	8.44 ± 2.22	8.89 ± 1.39

TABLE II-19: MUTAGENICITY ASSAY RESULTS OF DAGN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		54.33 ± 3.84	48.89 ± 4.55	200.33 ± 28.00	155.56 ± 8.77	220.78 ± 25.48	255.11 ± 28.01	22.11 ± 10.36	24.44 ± 5.42	18.33 ± 3.38	11.22 ± 4.25
DMSO		53.00 ± 10.04	38.89 ± 2.78	162.78 ± 11.65	146.89 ± 25.50	322.56 ± 44.89	239.22 ± 16.23	16.78 ± 1.84	20.33 ± 4.63	17.67 ± 5.51	15.44 ± 3.53
Anthramine		1547.00 ± 197.56		1832.22 ± 153.61		414.33 ± 47.71		178.44 ± 18.47		196.33 ± 45.00	
Aminoacridine											210.67 ± 16.09
Mitomycin C							716.56 ± 99.55				
2-Nitrofluorene			782.44 ± 43.87								
Sodium azide					639.78 ± 55.23				493.78 ± 9.35		
DAGN (mg)	(mM)										
0.03	0.2			179.33 ± 35.18	191.89 ± 33.13	168.33 ± 72.28	236.78 ± 47.23	11.22 ± 0.96	15.22 ± 1.84		
0.1	0.54	58.33 ± 10.99	37.67 ± 9.71	118.22 ± 31.48	163.78 ± 19.68	305.67 ± 105.69	285.33 ± 29.14	16.89 ± 4.48	14.11 ± 4.44	13.67 ± 4.04	12.00 ± 4.58
0.3	2.0	61.11 ± 5.75	32.00 ± 6.57	116.33 ± 26.69	179.56 ± 30.65	333.89 ± 27.36	235.22 ± 63.27	14.56 ± 1.95	15.00 ± 0.00	12.00 ± 4.33	8.11 ± 2.50
1	5.4	44.67 ± 14.44	46.56 ± 12.69	94.33 ± 5.61	148.89 ± 29.19	312.22 ± 32.27	296.00 ± 13.68	16.22 ± 4.55	17.00 ± 0.67	8.44 ± 0.84	8.67 ± 2.33
3	20	35.11 ± 4.00	31.00 ± 4.36	125.11 ± 23.06	85.00 ± 102.50	267.56 ± 8.69	197.11 ± 28.77	20.89 ± 6.77	1.22 ± 2.12	15.78 ± 6.83	8.22 ± 2.27
5	32	28.11 ± 3.75	25.67 ± 0.58							5.44 ± 0.51	0.00 ± 0.00

TABLE II-20: MUTAGENICITY ASSAY RESULTS OF NAGN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		30.78 ± 2.83	32.67 ± 2.91	133.00 ± 14.88	140.67 ± 8.97	305.56 ± 23.43	274.56 ± 19.15	15.00 ± 3.53	12.67 ± 2.19	13.00 ± 2.33	8.67 ± 1.20
DMSO		30.44 ± 10.34	24.00 ± 5.24	136.56 ± 10.82	133.22 ± 3.67	316.22 ± 29.76	267.67 ± 32.26	12.56 ± 4.44	14.44 ± 1.35	9.11 ± 2.04	12.44 ± 1.54
Anthramine		1021.33 ± 96.74		1751.67 ± 194.01		324.22 ± 39.17		126.00 ± 17.64		133.44 ± 25.93	
Aminoacridine											151.11 ± 42.59
Mitomycin C							1052.67 ± 52.34				
2-Nitrofluorene			593.56 ± 103.26								
Sodium azide					786.44 ± 31.64				746.67 ± 11.33		
NAGN (mg)	(mM)										
0.03	0.16	38.00 ± 4.58	24.00 ± 5.77	147.67 ± 6.56	142.33 ± 11.15	312.78 ± 22.79	277.00 ± 10.97	11.78 ± 2.67	12.11 ± 5.19	7.56 ± 2.69	12.67 ± 1.33
0.1	0.5	32.89 ± 5.74	20.11 ± 4.79	140.56 ± 8.39	141.56 ± 1.26	315.33 ± 15.18	269.22 ± 14.73	13.00 ± 3.18	9.44 ± 2.50	10.89 ± 0.51	11.22 ± 2.91
0.3	1.6	33.67 ± 6.39	30.33 ± 8.02	137.89 ± 6.91	147.00 ± 18.67	317.33 ± 23.95	275.56 ± 20.38	12.67 ± 5.81	16.67 ± 6.98	16.44 ± 7.86	12.78 ± 3.17
1	5.4	32.00 ± 5.36	25.67 ± 3.53	127.11 ± 10.36	108.11 ± 9.64	319.11 ± 5.74	250.33 ± 29.02	12.44 ± 5.40	11.67 ± 4.63	11.56 ± 4.43	10.67 ± 1.20
3	16	29.22 ± 9.45	175.33 ± 97.06	126.11 ± 17.99	35.44 ± 40.54	299.78 ± 15.49	94.22 ± 20.67	14.89 ± 8.47	0.00 ± 0.00	7.67 ± 4.33	6.33 ± 1.53

TABLE II-21: MUTAGENICITY ASSAY RESULTS OF EAN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		22.78 ± 3.66	21.33 ± 6.03	121.22 ± 20.24	127.22 ± 4.72	288.56 ± 22.79	256.56 ± 19.55	18.78 ± 5.98	21.78 ± 7.38	12.89 ± 1.26	8.89 ± 2.83
DMSO		25.44 ± 2.99	25.56 ± 4.44	106.89 ± 7.32	105.22 ± 13.57	231.11 ± 36.22	250.67 ± 32.36	15.67 ± 2.91	14.33 ± 4.18	9.00 ± 0.33	8.67 ± 3.28
Anthramine		135.33 ± 77.95		836.00 ± 47.76		492.78 ± 19.35		166.22 ± 26.27		180.33 ± 34.18	
Aminoacridine											183.22 ± 40.47
Mitomycin C							918.22 ± 23.35				
2-Nitrofluorene			502.33 ± 37.47								
Sodium azide					470.11 ± 53.50				463.33 ± 13.74		
EAN (mg)	(mM)										
0.1	0.8	26.33 ± 2.60	16.00 ± 4.04	124.89 ± 19.27	121.56 ± 10.65	272.56 ± 20.06	235.22 ± 39.58	23.44 ± 5.21	17.33 ± 5.84	13.00 ± 2.91	10.33 ± 2.33
0.3	2.4	32.00 ± 7.51	21.44 ± 1.84	123.78 ± 12.19	115.11 ± 12.62	264.00 ± 8.89	228.33 ± 40.78	24.44 ± 3.98	14.78 ± 3.02	9.22 ± 2.14	11.67 ± 7.02
1	8	32.11 ± 1.17	24.78 ± 4.74	115.89 ± 7.38	120.67 ± 6.81	286.00 ± 9.24	220.89 ± 23.57	16.22 ± 3.34	12.11 ± 2.55	9.00 ± 2.60	10.89 ± 0.77
3	24	27.11 ± 7.60	18.33 ± 3.06	117.67 ± 14.26	100.44 ± 8.34	215.00 ± 9.77	187.89 ± 17.39	22.22 ± 8.28	17.56 ± 4.29	9.67 ± 1.86	6.78 ± 0.51
5	40	22.22 ± 5.58	16.56 ± 0.77	105.56 ± 6.62	91.89 ± 11.48	278.67 ± 53.13	233.22 ± 33.10	23.56 ± 10.49	17.00 ± 7.94	12.89 ± 1.84	8.78 ± 2.50

TABLE II-22: MUTAGENICITY ASSAY RESULTS OF TN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		44.67 ± 3.06	32.56 ± 4.02	156.44 ± 10.73	164.11 ± 5.68	348.11 ± 63.19	275.78 ± 20.66	11.11 ± 1.17	12.89 ± 0.69	14.11 ± 1.84	9.33 ± 3.48
DMSO		44.56 ± 4.30	25.22 ± 2.14	143.11 ± 20.64	150.22 ± 17.26	335.33 ± 5.51	276.44 ± 17.72	13.11 ± 4.88	13.56 ± 5.17	12.00 ± 0.88	8.00 ± 2.19
Anthramine		1149.56 ± 86.81		1518.78 ± 194.51		604.33 ± 15.31		199.00 ± 20.88		195.89 ± 20.44	
Aminoacridine											182.00 ± 29.45
Mitomycin C							1014.89 ± 10.51				
2-Nitrofluorene			575.44 ± 22.97								
Sodium azide					756.78 ± 83.11				642.22 ± 34.00		
TN (mg)	(mM)										
0.1	0.75	33.00 ± 3.18	21.78 ± 1.84	162.67 ± 16.95	161.67 ± 6.00	341.33 ± 44.38	268.44 ± 6.74	8.56 ± 0.38	15.44 ± 3.15	9.78 ± 3.83	5.56 ± 1.64
0.3	2.2	41.11 ± 8.04	23.22 ± 7.12	169.44 ± 18.34	148.78 ± 4.40	325.00 ± 12.22	276.44 ± 19.02	13.11 ± 7.50	15.44 ± 6.35	8.11 ± 2.67	8.00 ± 4.48
1	7.5	44.11 ± 6.48	19.56 ± 7.50	165.00 ± 10.97	99.44 ± 14.85	321.78 ± 13.68	168.00 ± 10.48	8.56 ± 0.51	9.33 ± 4.26	10.78 ± 2.83	7.22 ± 0.84
3	22	34.56 ± 8.67	0.00 ± 0.00	132.67 ± 15.30	0.00 ± 0.00	284.56 ± 20.39	38.67 ± 2.73	7.44 ± 1.71	0.00 ± 0.00	12.89 ± 0.51	0.00 ± 0.00
5	38	5.44 ± 1.50	0.00 ± 0.00	61.11 ± 3.69	0.00 ± 0.00	39.00 ± 2.33	71.67 ± 7.64	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

TABLE II-23: MUTAGENICITY ASSAY RESULTS OF ATN

Treatment		TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous		27.67 ± 11.89	29.56 ± 6.19	153.00 ± 18.48	162.22 ± 10.08	248.11 ± 41.64	304.56 ± 38.79	8.56 ± 1.54	12.11 ± 3.66	8.00 ± 3.38	9.44 ± 3.75
DMSO		40.44 ± 3.01	27.11 ± 4.02	140.44 ± 34.00	156.89 ± 13.81	263.78 ± 22.71	261.67 ± 30.87	10.89 ± 2.59	12.00 ± 5.00	9.89 ± 4.48	6.67 ± 1.20
Anthramine		1015.67 ± 108.38		1414.00 ± 169.75		456.89 ± 13.61		229.11 ± 8.76		223.44 ± 9.48	
Aminoacridine											136.44 ± 18.86
Mitomycin C							791.33 ± 68.06				
2-Nitrofluorene			817.89 ± 102.42								
Sodium azide					654.33 ± 27.50				655.33 ± 52.20		
ATN (mg)	(mM)										
0.1	0.67	29.00 ± 1.33	24.44 ± 4.68	151.22 ± 27.27	138.67 ± 19.73	293.78 ± 15.70	259.89 ± 2.67	9.33 ± 1.33	16.56 ± 2.67	12.00 ± 0.88	10.89 ± 2.17
0.3	2.0	27.67 ± 4.18	33.11 ± 7.82	168.89 ± 23.12	155.67 ± 8.41	255.00 ± 13.50	256.89 ± 15.03	7.78 ± 0.51	11.33 ± 2.52	11.44 ± 0.84	11.56 ± 2.22
1	6.7	26.56 ± 0.69	30.89 ± 1.39	165.56 ± 13.50	135.67 ± 3.84	265.89 ± 6.59	223.33 ± 2.00	7.00 ± 1.45	4.78 ± 4.17	11.44 ± 2.50	8.89 ± 2.01
3	20	38.00 ± 19.92	32.89 ± 25.24	116.78 ± 39.50	91.44 ± 18.31	184.67 ± 7.00	230.22 ± 25.24	55.56 ± 25.13	0.00 ± 0.00	10.56 ± 3.72	6.44 ± 3.29
5	34	38.22 ± 23.25	0.00 ± 0.00	2.56 ± 4.43	0.00 ± 0.00	172.33 ± 33.39	85.22 ± 15.49	1.67 ± 1.53	0.00 ± 0.00	3.33 ± 0.58	0.00 ± 0.00

TABLE II-24: EXPERIMENTAL SUMMARY OF 11 HIGH ENERGY CHEMICALS

Chemicals	Ames Test Toxicity	Mutagenicity	Site of Mutation	Strain #	Type of Mutation*
HZN	Moderate toxicity	Positive[a]	AT and GC base- pair substitution	TA102 and TA1535	Indirect
DEHN	Moderate toxicity	NA**			
DHTN	Severe toxicity	Positive	AT and GC base- pair substitution frameshift mutation	TA98, TA102 and TA1535	Direct
DAGN	Minimal toxicity	Negative			
NAGN	Minimal toxicity	Negative			
EAN	Minimal toxicity	Negative			
HDN	Minimal toxicity	Weak[c]	AT base - pair substitution	TA102	Direct
MAN	Moderate toxicity	Moderate[b]	GC base - pair substitution	TA100	Indirect
TN	Moderate toxicity	Negative			
ATN	Minimal toxicity	Negative			
DMTN	Minimal toxicity	Weak	AT base - pair substitution	TA102	Direct

* Indirect required S9 mixture for metabolic activation

** Only dose selection assay was performed

[a]Positive: 1. Good dose response relationship
2. More than one strain showed dose response relationship
3. More than 2-fold induction

[b]Moderate: 1. Good dose response relationship
2. Only one strain showed dose response relationship
3. More than 2-fold induction

[c]Weak: 1. No dose response relationship; at least two concentrations showed induction
2. Only one strain showed a response
3. Less than 2-fold induction

DISCUSSION

In this study, we tested ten hydrazine derivatives and amino-containing high energy compounds for their mutagenicity in bacterial system using the pre-incubation method recommended for nitro compounds, a selective and sensitive method.

In the dose selection assay, we used 5 mg/plate as top dose which is the maximum dose recommended by EPA's Office of PoHution Prevention and Toxics (OPPTs) health effects guideline. The dose selection data showed that HZN, DHTN, NAGN, MAN and DMTN were more toxic to the tester strains compared with DEHN, DAGN, EAN HDN and ATN. Therefore, the doses selected for the mutagenesis assay were 0.03-3.0 mg/plate for HZN, NAGN, DHTN, MAN, DMTN and 0.1–5.0 mg/plate for EAN, HDN, TN and ATN. In the case of DAGN, the test range was 0.03–3.0 mg/plate for TA100, TA102 and TA1535, 0.1–5.0 mg/plate for TA98 and TA1537. However, by using the above doses, HZN showed toxicity at 3 mg/plate, DHTN showed toxicity at 1 and 3 mg/plate, MAN and TN showed toxicity at 3.0 mg/plate. The other chemicals did not show any toxicity or slight toxicity in the mutagenesis assay. Combining the toxicity data from the two types of experiments (dose selection and mutagenicity), it was observed that the top dose (5 mg/plate, the maximum dose required for this assay) was extremely toxic. The ranking of agents based on toxicity is as follows: DHTN>HZN>MAN>TN>DEHN, DAGN, NAGN, EAN, HDN, ATN and DMTN.

The results from the mutagenesis assay indicated that HZN increased the revertants in TA102 and TA1535 with very good dose-dependent response in the presence of S9 system. The increase in the number of revertants was 1.2-1.4 fold and 2.0-5.0 fold, respectively, that of DMSO control. DHTN increased the revertants in TA98, TA100 and TA102 either in both with and without S9 activation system or without S9 system only (TA98). The increase in revertant numbers ranged from 1.5–3.7 fold compared with DMSO control. MAN significantly increased the revertants in TA100 with and without S9 activation system by 3.4–4.6 fold. HDN and DMTN slightly increased the numbers of revertants in TA102 at different concentrations. Among the five positive chemicals, four chemicals induced the mutant revertants in tester strain TA102, a strain that has AT base pair at the primary reversion site and has selected sensitivity to hydrazine compounds (Wilcox, 1990). In our previous study, hydroxyethylhydrazinium nitrate (HEHN, another hydrazine derivative) induced the revertants in TA102 and TA1535 with a very good dose response manner, and also increased the size of colonies in the TA1535 induced revertants (Sharma & Gao, 1999).

Interestingly, less toxicity was noticed in several chemicals at the high doses in the presence of S9 activation system compared with S9 negative system. It is possible that there is an increased detoxification of these compounds due to the presence of phase II enzymes that are present in the S9 fraction.

The above results under the experimental condition indicate that HZN, DHTN and MAN are mutagenic to bacteria, as it has caused an increase in the number of revertants in tester strains over three or more concentrations. Further, DHTN is a direct mutagen causing AT and GC base-pair substitutions and frameshift mutations as there was no difference in the numbers of revertants between the activated (S9+) and non-activated (S9-) group ($p>0.05$). Furthermore, HZN and DHTN increased the revertants in multiple strains including the sensitive TA102 strain

with induction up to 4 fold. On the other hand, MAN, one of the amino compounds, induced the mutant revertants in TA100 only (and not in the sensitive strain, TA102). HZN and MAN are indirect mutagens, as there is a difference between S9+ and S9- group ($p<0.05$). Finally, HDN and DMTN are considered weak mutagens, since the increased number of revertants did not reach the standard criteria of 2 fold increase over the solvent control, however, the induction of revertants is still considered statistically significant. An experimental summary of all tested chemicals is included in Table II-24.

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APPENDIX II-A

Raw Data of *Salmonella* / Microsome Mutagenesis Assay

TABLE II-A1: MUTAGENICITY ASSAY RESULTS OF HZN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	#2	23	18	131	83	161	167	10	14	11	5
	#2	24	18	93	86	208	181	7	18	13	9
	#3	18	17	117	103	197	149	7	18	9	6
	Mean	21.89	17.56	113.67	90.78	188.33	165.89	8.00	16.67	10.89	6.78
	SD	3.15	0.51	19.34	10.94	24.58	15.88	2.03	2.33	2.17	1.71
DMSO	#2	21	20	92	119	266	228	15	10	13	10
	#2	20	25	111	109	268	232	11	13	12	8
	#3	23	14	104	114	253	242	14	13	10	8
	Mean	21.11	19.78	102.33	114.00	262.22	233.67	13.22	12.00	11.78	8.89
	SD	1.39	5.19	9.94	5.33	8.03	7.21	2.27	1.73	1.26	0.96
Positive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Control	#2	830	410	1232	358	756	1311	170	449	209	114
	#2	844	352	1019	580	652	1320	184	481	207	141
	#3	784	379	1232	554	545	1309	193	496	203	132
	Mean	819.33	380	1160.89	497.33	650.89	1313.11	182.11	475.11	206.44	128.89
	SD	31.15	29.02	122.88	121.1	105.51	5.8	11.74	24.05	3.2	13.93
HZN (mg / plate)											
0.03	#2	21	27	114	111	269	194	28	19	10	8
	#2	34	17	116	103	259	218	26	19	7	8
	#3	27	17	146	94	238	218	32	13	9	10
	Mean	27.33	20.33	125.33	102.67	255.44	210.00	28.78	16.89	8.67	8.89
	SD	6.84	5.77	17.93	8.35	15.54	14.15	2.69	3.67	1.76	1.26
0.1	#2	33	12	111	102	303	197	50	12	7	11
	#2	21	19	134	101	296	218	42	19	13	8
	#3	23	16	128	110	270	217	44	17	9	5
	Mean	26.00	15.56	124.22	104.33	289.89	210.67	45.11	16.11	9.56	7.89
	SD	6.43	3.36	12.20	5.21	17.26	11.55	4.11	3.34	2.78	3.02
0.3	#2	27	11	125	106	324	265	65	11	10	6
	#2	26	7	131	130	326	221	62	10	9	7
	#3	29	7	142	120	322	261	54	15	6	8
	Mean	27.67	8.33	132.56	118.78	323.89	248.78	60.22	12.00	8.44	6.89

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	1.53	2.33	8.77	12.21	1.84	24.41	5.55	2.65	2.22	0.84
1	#2	18	0	141	0	317	0	99	4	9	0
	#2	18	0	132	0	350	0	65	2	13	0
	#3	26	0	136	0	374	0	66	2	13	0
	Mean	20.67	0.00	136.56	0.00	347.11	0.00	76.67	2.67	11.56	0.00
	SD	4.33	0.00	4.67	0.00	28.97	0.00	19.35	1.15	2.22	0.00
3	#2	8	0	51	0	177	0	37	0	6	0
	#2	10	0	46	0	227	0	34	0	8	0
	#3	8	0	44	0	216	0	50	0	4	0
	Mean	8.78	0.00	46.78	0.00	206.67	0.00	40.33	0.00	6.11	0.00
	SD	1.07	0.00	3.56	0.00	26.35	0.00	8.45	0.00	1.84	0.00

TABLE II-A2: MUTAGENICITY ASSAY RESULTS OF DHTN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	#1	15	35	155	160	366	214	12	4	11	13
	#2	41	44	125	143	332	314	15	10	13	11
	#3	27	21	162	159	333	230	10	20	10	13
	Mean	27.67	33.44	147.22	154.11	343.78	252.56	12.33	11.56	11.33	12.22
	SD	13.17	11.41	19.79	9.65	18.96	53.53	2.40	7.90	1.45	0.77
DMSO	#1	20	30	125	136	242	227	10	7	20	5
	#2	35	25	132	111	250	262	7	14	17	8
	#3	40	22	140	102	339	222	9	11	11	12
	Mean	31.78	25.33	132.11	116.11	277.22	236.78	8.89	10.78	16.00	8.11
	SD	10.46	4.04	7.50	17.48	53.93	21.99	1.50	3.86	4.26	3.36
Positive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Control	#1	1089	501	1768	743	635	985	222	526	344	107
	#2	1247	492	1556	697	680	858	173	539	308	175
	#3	936	461	2016	667	544	874	185	526	326	212
	Mean	1090.56	484.67	1780	702.67	619.78	905.89	193.22	530.22	325.78	164.33
	SD	155.5	21.3	229.9	38.28	69.65	69.28	25.6	7.89	17.83	53.26
DHTN (mg / plate)											
0.03	#1	62	45	283	276	428	411	17	21	12	15
	#2	76	47	266	242	479	455	14	19	13	13
	#3	62	65	204	239	369	409	25	13	16	8
	Mean	66.56	52.44	251.11	252.33	425.44	425.22	18.56	17.67	13.78	12.00
	SD	7.90	11.18	41.96	20.58	54.72	26.10	5.82	3.93	1.71	3.84
0.1	#1	48	73	418	332	553	424	23	15	14	17
	#2	59	69	541	378	518	462	42	22	14	13
	#3	52	66	383	496	482	424	22	28	13	9
	Mean	52.89	69.33	447.33	401.89	517.56	436.56	28.78	21.78	13.67	13.11
	SD	5.52	3.84	82.67	84.96	35.83	22.04	11.46	6.52	0.67	4.17
0.3	#1	70	47	578	227	492	400	39	4	12	7
	#2	44	42	456	168	476	469	49	11	19	7
	#3	41	51	432	241	518	396	42	10	10	10
	Mean	51.78	46.78	488.78	212.11	495.22	421.67	43.56	8.33	13.67	8.11

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	15.87	4.34	78.53	39.12	21.55	41.06	4.95	3.48	4.81	1.64
1	#1	0	0	0	0	440	0	0	0	20	0
	#2	26	0	0	0	414	0	0	0	12	0
	#3	21	0	0	0	429	0	0	0	13	0
	Mean	15.56	0.00	0.00	0.00	427.67	0.00	0.00	0.00	15.22	0.00
	SD	13.73	0.00	0.00	0.00	12.86	0.00	0.00	0.00	4.48	0.00
3	#1	0	0	0	0	0	0	0	0	0	0
	#2	0	0	0	0	0	0	0	0	0	0
	#3	0	0	0	0	0	0	0	0	0	0
	Mean	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

TABLE II-A3: MUTAGENICITY ASSAY RESULTS OF HDN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	#1	47	41	163	174	303	284	14	16	11	18
	#2	66	45	164	155	392	290	12	15	9	10
	#3	62	45	132	158	375	245	13	18	11	14
	Mean	58.56	43.78	153.00	162.22	356.78	273.22	13.22	16.33	10.33	13.78
	SD	9.89	2.14	18.48	10.08	47.00	24.62	1.02	1.67	1.15	4.17
DMSO	#1	62	36	174	147	314	242	15	15	18	14
	#2	46	36	106	173	317	251	13	20	10	11
	#3	57	43	141	151	273	289	14	13	9	15
	Mean	54.89	38.33	140.44	156.89	301.11	260.33	14.00	16.11	12.56	13.33
	SD	8.04	3.76	34.00	13.81	24.69	24.95	1.20	3.79	5.05	2.08
Positive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Conrol	#1	1613	496	1220	648	646	1088	123	630	102	132
	#2	1250	449	1537	684	501	1104	136	591	103	56
	#3	1019	477	1485	630	503	1144	120	565	109	138
	Mean	1293.89	473.89	1414	654.33	550.11	1112.11	126.44	595.44	104.67	109
	SD	299.46	23.82	169.75	27.5	83.05	28.47	8.13	32.36	3.48	45.71
HDN (mg/ plate)											
0.1	#1	54	34	152	134	337	275	14	13	22	10
	#2	47	46	158	155	354	309	20	13	13	12
	#3	50	38	156	141	331	223	19	21	10	11
	Mean	50.56	39.44	155.33	143.33	340.78	269.00	17.89	15.67	14.78	10.89
	SD	3.67	6.19	3.28	10.48	11.80	43.18	3.40	4.91	6.19	0.69
0.3	#1	41	32	155	142	277	305	12	15	13	9
	#2	54	33	171	149	314	294	24	15	18	16
	#3	57	41	132	164	401	273	12	13	8	11
	Mean	50.78	35.33	152.56	151.78	330.44	290.56	16.11	14.22	13.11	11.89
	SD	8.32	4.93	19.25	10.87	63.84	16.55	6.54	1.07	5.17	3.42
1	#1	45	37	138	139	345	255	15	6	19	11
	#2	39	33	154	163	265	277	16	17	14	11
	#3	34	33	162	151	354	244	15	16	10	9
	Mean	39.44	34.33	151.22	150.89	321.44	258.56	15.33	12.78	14.67	10.44

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	5.17	2.60	12.51	11.67	49.38	16.84	0.33	6.19	4.51	1.26
3	#1	43	49	146	156	386	263	10	14	15	10
	#2	45	36	176	161	400	269	9	16	14	18
	#3	37	50	165	170	367	271	8	12	14	10
	Mean	41.67	45.11	162.44	162.22	384.44	267.33	9.22	14.22	14.33	12.78
	SD	4.16	7.62	15.33	7.46	16.58	4.16	1.02	1.84	0.33	4.53
5	#1	50	34	164	159	492	377	14	12	20	10
	#2	40	38	160	170	496	417	12	9	16	8
	#3	38	37	187	154	516	400	20	17	16	9
	Mean	42.56	36.33	170.56	161.00	501.33	398.00	15.44	12.67	17.56	9.00
	SD	6.50	2.40	14.38	8.51	12.55	19.94	4.35	3.71	2.41	0.88

TABLE II-A4: MUTAGENICITY ASSAY RESULTS OF MAN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	#1	38	33	121	141	323	321	21	16	16	17
	#2	36	28	97	134	303	329	9	9	8	15
	#3	40	33	103	204	345	307	16	10	18	11
	Mean	38.11	31.11	107.11	159.78	323.44	318.89	15.33	11.67	14.22	14.22
	SD	1.84	2.99	12.76	38.72	21.18	10.99	6.17	4.06	5.23	3.24
DMSO	#1	28	25	273	215	303	268	8	12	11	8
	#2	37	28	311	287	302	243	11	15	13	10
	#3	49	41	243	223	252	229	9	6	21	13
	Mean	38.22	31.44	275.56	241.78	285.44	246.67	9.44	11.00	14.67	10.56
	SD	10.69	8.44	33.76	39.08	29.25	19.64	1.17	4.26	5.29[1]	2.67
Positive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Control	#1	439	368	1292	950	402	875	90	603	148	86
	#2	650	346	1331	887	367	994	93	525	166	164
	#3	707	328	1157	995	399	909	132	611	129	160
	Mean	598.44	347.33	1260	944.33	389.22	926.11	105	579.78	147.67	136.67
	SD	140.99	20.22	90.99	54.25	19.62	61.48	23.41	47.89	18.5	43.92
MAN (mg / plate)											
0.03	#1	30	31	1315	1106	294	238	13	11	11	12
	#2	32	36	1294	1092	203	252	13	7	8	9
	#3	44	28	1231	945	385	317	17	11	16	6
	Mean	35.11	32.00	1279.89	1047.78	294.00	269.00	14.56	9.56	11.56	8.89
	SD	7.76	4.04	43.66	89.30	91.00	42.15	2.12	1.92	4.07	2.67
0.1	#1	64	49	1178	1095	346	257	13	7	11	17
	#2	43	41	1285	1108	340	229	9	5	12	9
	#3	43	39	1216	940	340	261	8	10	13	6
	Mean	50.22	42.78	1226.22	1047.56	341.89	249.00	10.22	7.33	12.00	10.56
	SD	12.22	5.23	54.18	93.08	3.56	17.44	2.71	2.19	1.00	5.50
0.3	#1	23	32	1241	1126	301	270	7	17	13	13
	#2	33	29	1276	1074	303	289	22	9	10	6
	#3	24	28	1231	1063	338	300	12	15	8	12
	Mean	26.67	29.78	1249.44	1087.67	314.00	286.33	13.44	13.56	10.33	10.44

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	5.49	2.27	23.57	33.71	21.11	15.18	7.49	4.35	2.85	3.56
1	#1	31	27	1265	764	283	188	35	14	9	0
	#2	24	25	1306	828	259	191	11	19	3	10
	#3	30	21	1265	801	265	236	22	27	7	5
	Mean	28.33	24.22	1278.78	797.44	268.89	204.89	22.78	20.00	6.33	5.00
	SD	3.79	3.24	23.87	32.15	12.54	26.70	11.67	6.23	3.06	4.84
3	#1	5	0	1208	63	0	0	4	0	3	0
	#2	0	0	1201	5	0	0	10	0	2	6
	#3	3	0	1231	30	0	0	4	0	0	0
	Mean	2.67	0.00	1213.33	32.67	0.00	0.00	6.00	0.00	1.56	2.11
	SD	2.67	0.00	15.70	29.42	0.00	0.00	3.76	0.00	1.39	3.66

TABLE II-A5: MUTAGENICITY ASSAY RESULTS OF DMTN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	#1	31	33	163	174	239	173	24	15	10	10
	#2	31	34	164	155	209	205	22	13	10	12
	#3	33	30	132	158	214	190	11	15	5	9
	Mean	31.56	32.00	153.00	162.22	220.67	189.33	18.89	14.22	8.33	10.33
	SD	0.96	2.08	18.48	10.08	16.38	15.68	7.24	1.07	3.18	1.86
DMSO	#1	25	23	174	147	234	156	14	9	11	8
	#2	26	22	106	173	292	209	22	14	14	11
	#3	29	21	141	151	281	227	14	11	11	7
	Mean	26.78	22.11	140.44	156.89	268.78	197.11	16.67	11.33	11.89	8.67
	SD	1.71	0.84	34.00	13.81	30.93	36.72	4.62	2.33	1.58	2.33
Posiive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Conrol	#1	843	511	1220	648	298	827	77	192	52	136
	#2	806	610	1537	684	263	934	102	207	103	114
	#3	955	638	1485	630	261	923	134	167	75	72
	Mean	868	586.67	1414	654.33	273.89	895	104.56	188.67	76.78	107.33
	SD	77.66	66.73	169.75	27.5	20.6	58.86	28.59	20.33	25.71	32.52
DMTN (mg/ plate)											
0.03	#1	45	32	147	164	402	303	29	12	6	12
	#2	46	36	153	173	361	306	19	19	18	8
	#3	29	45	148	154	283	315	14	15	22	15
	Mean	40.00	37.44	149.11	164.00	348.56	308.00	20.56	15.44	15.67	11.44
	SD	9.26	6.84	3.15	9.50	60.63	6.33	8.00	3.56	8.33	3.34
0.1	#1	30	47	181	155	361	289	11	14	6	10
	#2	33	42	161	135	330	257	13	11	17	12
	#3	44	51	172	165	378	308	19	14	12	9
	Mean	35.67	46.78	171.44	151.33	356.33	284.78	14.33	13.00	11.67	10.33
	SD	7.69	4.67	10.03	15.28	24.67	25.93	3.93	1.76	5.33	1.53
0.3	#1	54	47	173	154	326	295	10	17	10	8
	#2	51	36	179	165	368	293	9	11	9	15
	#3	49	46	153	146	368	287	16	11	11	11
	Mean	51.22	43.00	168.44	155.22	354.11	291.56	11.44	13.11	10.00	11.44

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	2.67	5.78	13.50	9.70	24.35	4.03	3.98	3.37	1.20	3.36
1	#1	47	44	146	142	393	300	13	12	9	10
	#2	38	51	160	174	284	268	22	12	8	11
	#3	47	56	151	156	372	316	14	10	8	10
	Mean	43.67	50.22	152.56	157.22	349.78	294.33	16.22	11.11	8.22	10.22
	SD	5.20	6.08	7.24	15.72	57.58	24.44	4.74	1.26	0.96	0.38
3	#1	38	41	196	106	424	353	10	7	9	9
	#2	40	44	182	106	397	315	15	8	6	7
	#3	47	34	203	115	322	329	8	14	10	10
	Mean	41.44	39.67	193.44	109.00	381.33	332.33	11.00	9.67	8.44	8.89
	SD	4.67	5.24	10.51	4.91	52.85	19.34	3.93	3.53	2.22	1.39

TABLE II-A6: MUTAGENICITY ASSAY RESULTS OF DAGN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Sponaneous	#1	54	50	184	165	239	227	19	20	18	13
	#2	58	53	233	148	232	256	34	23	22	14
	#3	51	44	184	154	192	283	14	30	15	6
	Mean	54.33	48.89	200.33	155.56	220.78	255.11	22.11	24.44	18.33	11.22
	SD	3.84	4.55	28.00	8.77	25.48	28.01	10.36	5.42	3.38	4.25
DMSO	#1	51	38	176	172	329	254	18	17	14	12
	#2	44	37	160	147	275	222	15	18	15	16
	#3	64	42	153	121	364	242	18	26	24	19
	Mean	53.00	38.89	162.78	146.89	322.56	239.22	16.78	20.33	17.67	15.44
	SD	10.04	2.78	11.65	25.50	44.89	16.23	1.84	4.63	5.51	3.53
Positive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Contol	#1	1533	812	1662	588	455	602	185	492	210	216
	#2	1357	804	1960	634	362	771	193	486	233	193
	#3	1751	732	1875	698	427	777	158	504	146	224
	Mean	1547	782.44	1832.22	639.78	414.33	716.56	178.44	493.78	196.33	210.67
	SD	197.56	43.87	153.61	55.23	47.71	99.55	18.47	9.35	45	16.09
DAGN (mg /plate)											
0.03	#1			189	156	123	285	11	13		
	#2			209	221	252	234	12	15		
	#3			140	199	131	191	11	17		
	Mean			179.33	191.89	168.33	236.78	11.22	15.22		
	SD			35.18	33.13	72.28	47.23	0.96	1.84		
0.1	#1	53	48	153	181	184	255	22	16	17	13
	#2	51	35	110	142	369	313	14	17	14	7
	#3	71	29	92	168	364	289	15	9	9	16
	Mean	58.33	37.67	118.22	163.78	305.67	285.33	16.89	14.11	13.67	12.00
	SD	10.99	9.71	31.48	19.68	105.69	29.14	4.48	4.44	4.04	4.58
0.3	#1	56	27	142	207	339	162	15	15	9	11
	#2	60	39	118	147	304	276	12	15	10	7
	#3	67	30	89	185	358	267	16	15	17	7
	Mean	61.11	32.00	116.33	179.56	333.89	235.22	14.56	15.00	12.00	8.11
	SD	5.75	6.57	26.69	30.65	27.36	63.27	1.95	0.00	4.33	2.50
1	#1	61	60	101	148	277	310	17	18	8	11
	#2	36	35	90	179	340	283	20	16	8	7
	#3	37	44	92	120	320	295	11	17	9	8
	Mean	44.67	46.56	94.33	148.89	312.22	296.00	16.22	17.00	8.44	8.67

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	14.44	12.69	5.61	29.19	32.27	13.68	4.55	0.67	0.84	2.33
3	#1	39	28	141	47	258	166	16	4	9	6
	#2	35	36	136	7	271	202	29	0	16	10
	#3	31	29	99	201	274	223	18	0	23	9
	Mean	35.11	31.00	125.11	85.00	267.56	197.11	20.89	1.22	15.78	8.22
	SD	4.00	4.36	23.06	102.50	8.69	28.77	6.77	2.12	6.83	2.27
5	#1	29	26							5	0
	#2	24	25							5	0
	#3	31	26							6	0
	Mean	28.11	25.67							5.44	0.00
	SD	3.75	0.58							0.51	0.00

TABLE II-A7: MUTAGENICITY ASSAY RESULTS OF NAGN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	#1	34	29	139	151	328	264	19	10	14	10
	#2	30	34	116	138	308	263	14	13	15	9
	#3	29	35	144	133	281	297	12	15	10	7
	Mean	30.78	32.67	133.00	140.67	305.56	274.56	15.00	12.67	13.00	8.67
	SD	2.83	2.91	14.88	8.97	23.43	19.15	3.53	2.19	2.33	1.20
DMSC	#1	31	29	149	133	282	292	8	15	9	11
	#2	41	25	129	137	336	231	14	16	11	13
	#3	20	18	131	130	331	280	16	13	7	13
	Mean	30.44	24.00	136.56	133.22	316.22	267.67	12.56	14.44	9.11	12.44
	SD	10.34	5.24	10.82	3.67	29.76	32.26	4.44	1.35	2.04	1.54
Positive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Control	#1	910	602	1912	820	365	1052	133	747	155	103
	#2	1080	692	1807	757	287	1105	106	758	141	166
	#3	1075	486	1536	783	320	1001	139	735	105	184
	Mean	1021.33	593.56	1751.67	786.44	324.22	1052.67	126	746.67	133.44	151.11
	SD	96.74	103.26	194.01	31.64	39.17	52.34	17.64	11.33	25.93	42.59
NAGN (mg / plate)											
0.03	#1	34	31	155	130	294	289	12	17	8	11
	#2	43	21	142	151	338	273	14	13	5	14
	#3	37	21	147	147	307	268	9	7	10	13
	Mean	38.00	24.00	147.67	142.33	312.78	277.00	11.78	12.11	7.56	12.67
	SD	4.58	5.77	6.56	11.15	22.79	10.97	2.67	5.19	2.69	1.33
0.1	#1	31	15	148	143	332	255	11	12	11	14
	#2	39	24	132	141	302	285	11	8	11	12
	#3	28	22	142	141	313	268	17	8	10	8
	Mean	32.89	20.11	140.56	141.56	315.33	269.22	13.00	9.44	10.89	11.22
	SD	5.74	4.79	8.39	1.26	15.18	14.73	3.18	2.50	0.51	2.91
0.3	#1	31	38	145	155	310	256	19	23	25	10
	#2	41	22	132	160	298	297	10	18	16	16
	#3	29	31	137	126	344	274	9	9	9	13
	Mean	33.67	30.33	137.89	147.00	317.33	275.56	12.67	16.67	16.44	12.78

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	6.39	8.02	6.91	18.67	23.95	20.38	5.81	6.98	7.86	3.17
1	#1	31	24	124	110	324	225	9	9	17	12
	#2	27	30	139	117	313	282	19	17	9	10
	#3	38	23	119	98	321	244	10	9	9	10
	Mean	32.00	25.67	127.11	108.11	319.11	250.33	12.44	11.67	11.56	10.67
	SD	5.36	3.53	10.36	9.64	5.74	29.02	5.40	4.63	4.43	1.20
3	#1	18	135	132	81	310	73	22	0	8	6
	#2	35	105	106	3	307	115	6	0	3	5
	#3	34	286	141	22	282	95	17	0	12	8
	Mean	29.22	175.33	126.11	35.44	299.78	94.22	14.89	0.00	7.67	6.33
	SD	9.45	97.06	17.99	40.54	15.49	20.67	8.47	0.00	4.33	1.53

TABLE II-A8: MUTAGENICITY ASSAY RESULTS OF EAN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Sportaneous	#1	19	22	113	133	270	267	26	13	12	11
	#2	24	27	144	124	314	234	16	25	14	6
	#3	26	15	107	125	282	269	15	27	12	10
	Mean	22.78	21.33	121.22	127.22	288.56	256.56	18.78	21.78	12.89	8.89
	SD	3.66	6.03	20.24	4.72	22.79	19.55	5.98	7.38	1.26	2.83
DMSO	#1	27	31	103	93	270	215	19	14	9	10
	#2	22	23	102	102	225	260	14	10	9	5
	#3	27	23	115	120	198	277	14	19	9	11
	Mean	25.44	25.56	106.89	105.22	231.11	250.67	15.67	14.33	9.00	8.67
	SD	2.99	4.44	7.32	13.57	36.22	32.36	2.91	4.18	0.33	3.28
Posiive		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Control	#1	830	472	782	471	472	891	148	448	148	140
	#2	760	490	855	416	510	930	154	473	216	190
	#3	916	544	871	523	497	933	196	469	176	220
	Mean	835.33	502.33	836	470.11	492.78	918.22	166.22	463.33	180.33	183.22
	SD	77.95	37.47	47.76	53.5	19.35	23.35	26.27	13.74	34.18	40.47
EAN (mg/ plate)											
0.1	#1	23	12	103	117	292	249	19	11	16	13
	#2	28	20	137	134	274	266	29	23	11	9
	#3	28	15	135	114	252	191	23	18	12	9
	Mean	26.33	16.00	124.89	121.56	272.56	235.22	23.44	17.33	13.00	10.33
	SD	2.60	4.04	19.27	10.65	20.06	39.58	5.21	5.84	2.91	2.33
0.3	#1	36	23	112	112	257	249	26	16	8	18
	#2	23	20	123	129	261	181	20	17	8	12
	#3	36	21	136	104	274	254	28	11	12	4
	Mean	32.00	21.44	123.78	115.11	264.00	228.33	24.44	14.78	9.22	11.67
	SD	7.51	1.84	12.19	12.62	8.89	40.78	3.98	3.02	2.14	7.02
1	#1	33	28	124	123	292	229	20	9	12	11
	#2	32	19	111	113	291	194	13	13	8	11
	#3	31	27	113	126	275	239	16	14	7	10
	Mean	32.11	24.78	115.89	120.67	286.00	220.89	16.22	12.11	9.00	10.89

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	1.17	4.74	7.38	6.81	9.24	23.57	3.34	2.55	2.60	0.77
3	#1	31	15	134	92	207	185	27	19	9	6
	#2	32	21	111	100	226	172	13	21	8	7
	#3	18	19	108	109	212	207	27	13	12	7
	Mean	27.11	18.33	117.67	100.44	215.00	187.89	22.22	17.56	9.67	6.78
	SD	7.60	3.06	14.26	8.34	9.77	17.39	8.28	4.29	1.86	0.51
5	#1	19	17	98	98	217	271	17	11	13	6
	#2	19	17	108	99	310	219	36	26	11	11
	#3	29	16	110	79	308	209	18	14	15	9
	Mean	22.22	16.56	105.56	91.89	278.67	233.22	23.56	17.00	12.89	8.78
	SD	5.58	0.77	6.62	11.48	53.13	33.10	10.49	7.94	1.84	2.50

TABLE II-A9: MUTAGENICITY ASSAY RESULTS OF TN

Tratment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Sponaneous	#1	48	33	166	159	412	252	11	14	12	13
	#2	44	28	145	170	286	284	12	12	16	7
	#3	42	36	159	164	346	291	10	13	14	8
	Mean	44.67	32.56	156.44	164.11	348.11	275.78	11.11	12.89	14.11	9.33
	SD	3.06	4.02	10.73	5.68	63.19	20.66	1.17	0.69	1.84	3.48
DMSO	#1	41	24	156	166	341	256	12	8	12	8
	#2	43	28	119	132	330	287	9	17	11	10
	#3	49	24	154	152	336	286	18	16	13	6
	Mean	44.56	25.22	143.11	150.22	335.33	276.44	13.11	13.56	12.00	8.00
	SD	4.30	2.14	20.64	17.26	5.51	17.72	4.88	5.17	0.88	2.19
Positve		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
Contol	#1	1087	550	1597	663	593	1006	201	676	178	216
	#2	1249	582	1297	786	599	1026	177	642	191	164
	#3	1113	595	1662	821	622	1013	219	608	218	166
	Mean	1149.56	575.44	1518.78	756.78	604.33	1014.89	199	642.22	195.89	182
	SD	86.81	22.97	194.51	83.11	15.31	10.51	20.88	34	20.44	29.45
TN (mg plate)											
0.1	#1	35	23	181	162	388	261	9	13	10	4
	#2	29	20	160	168	336	272	8	19	14	6
	#3	35	23	147	156	300	272	8	14	6	7
	Mean	33.00	21.78	162.67	161.67	341.33	268.44	8.56	15.44	9.78	5.56
	SD	3.18	1.84	16.95	6.00	44.38	6.74	0.38	3.15	3.83	1.64
0.3	#1	39	18	162	144	338	258	13	22	8	4
	#2	50	31	190	153	314	296	21	9	11	13
	#3	34	20	156	150	322	275	6	15	5	7
	Mean	41.11	23.22	169.44	148.78	325.00	276.44	13.11	15.44	8.11	8.00
	SD	8.04	7.12	18.34	4.40	12.22	19.02	7.50	6.35	2.67	4.48
1	#1	37	14	159	85	329	156	9	10	8	6
	#2	50	17	178	115	330	173	9	13	11	7
	#3	46	28	159	99	306	175	8	5	14	8
	Mean	44.11	19.56	165.00	99.44	321.78	168.00	8.56	9.33	10.78	7.22

Tratment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	6.48	7.50	10.97	14.85	13.68	10.48	0.51	4.26	2.83	0.84
3	#1	26	0	150	0	304	36	6	0	13	0
	#2	34	0	124	0	286	41	9	0	12	0
	#3	43	0	124	0	263	39	7	0	13	0
	Mean	34.56	0.00	132.67	0.00	284.56	38.67	7.44	0.00	12.89	0.00
	SD	8.67	0.00	15.30	0.00	20.39	2.73	1.71	0.00	0.51	0.00
5	#1	7	0	65	0	38	78	0	0	0	0
	#2	5	0	61	0	42	73	0	0	0	0
	#3	4	0	58	0	37	63	0	0	0	0
	Mean	5.44	0.00	61.11	0.00	39.00	71.67	0.00	0.00	0.00	0.00
	SD	1.50	0.00	3.69	0.00	2.33	7.64	0.00	0.00	0.00	0.00

TABLE II-A10: MUTAGENICITY ASSAY RESULTS OF ATN

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
Spontaneous	#1	51	36	163	174	217	272	8	8	12	5
	#2	30	24	164	155	295	347	8	15	5	13
	#3	32	29	132	158	232	295	10	13	7	10
	Mean	37.67	29.56	153.00	162.22	248.11	304.56	8.56	12.11	8.00	9.44
	SD	11.89	6.19	18.48	10.08	41.64	38.79	1.54	3.66	3.38	3.75
DMSO	#1	43	23	174	147	238	263	8	7	15	5
	#2	37	27	106	173	271	230	13	17	7	8
	#3	41	31	141	151	282	292	12	12	8	7
	Mean	40.44	27.11	140.44	156.89	263.78	261.67	10.89	12.00	9.89	6.67
	SD	3.01	4.02	34.00	13.81	22.71	30.87	2.59	5.00	4.48	1.20
Positive Control		Anthramine	2-Nitrofluorene	Anthramine	Sodium azide	Anthramine	Mitomycin C	Anthramine	Sodium azide	Anthramine	Aminoacridine
	#1	1740	743	1220	648	447	736	222	620	234	157
	#2	1568	935	1537	684	472	867	239	630	221	121
	#3	1539	776	1485	630	452	771	226	715	216	131
	Mean	1615.67	817.89	1414	654.33	456.89	791.33	229.11	655.33	223.44	136.44
	SD	108.38	102.42	169.75	27.5	13.61	68.06	8.76	52.2	9.48	18.86
ATN (mg / plate)											
0.1	#1	28	29	159	152	310	260	8	14	13	9
	#2	29	20	121	116	279	257	9	19	12	11
	#3	30	24	174	148	293	263	11	16	11	13
	Mean	29.00	24.44	151.22	138.67	293.78	259.89	9.33	16.56	12.00	10.89
	SD	1.33	4.68	27.27	19.73	15.70	2.67	1.33	2.67	0.88	2.17
0.5	#1	24	26	151	150	268	264	8	14	11	14
	#2	32	42	195	152	241	240	7	9	11	10
	#3	27	31	161	165	255	267	8	11	12	11
	Mean	27.67	33.11	168.89	155.67	255.00	256.89	7.78	11.33	11.44	11.56
	SD	4.18	7.82	23.12	8.41	13.50	15.03	0.51	2.52	0.84	2.22
1	#1	36	29	178	133	260	223	5	8	14	7
	#2	37	32	151	140	265	221	8	0	9	11
	#3	36	31	168	134	273	225	8	7	11	9
	Mean	36.56	30.89	165.56	135.67	265.89	223.33	7.00	4.78	11.44	8.89

Treatment	Plate	TA98		TA100		TA102		TA1535		TA1537	
		S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-
	SD	0.69	1.39	13.50	3.84	6.59	2.00	1.45	4.17	2.50	2.01
3	#1	81	28	85	76	178	225	83	0	6	3
	#2	47	11	104	112	185	258	50	0	12	9
	#3	46	60	161	87	192	208	34	0	13	8
	Mean	58.00	32.89	116.78	91.44	184.78	230.22	55.56	0.00	10.56	6.44
	SD	19.92	25.24	39.50	18.31	6.83	25.24	25.13	0.00	3.72	3.29
5	#1	85	0	8	0	134	68	2	0	3	0
	#2	41	0	0	0	186	90	3	0	3	0
	#3	49	0	0	0	197	98	3	0	4	0
	Mean	58.22	0.00	2.56	0.00	172.33	85.22	1.67	0.00	3.33	0.00
	SD	23.25	0.00	4.43	0.00	33.39	15.49	1.53	0.00	0.58	0.00